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ACTION ON COLLAGEN OF NEUTRAL PROTEINASES PRODUCED BY CULTURED GINGIVAL EXPLANTS

by

HARVEY ALAN GOLDBERG

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A THESIS

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Produced by Cultured Gingival Explants

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ABSTRACT

Production of collagenase is a characteristic of many systems showing degradation of collagen. However it has been suggested that intermolecular cross-links in fibrillar collagen may present a significant obstacle to the action of collagenase. An activity that cleaves the telopeptides, which contain the cross-link sites, may be required as the first step in the turnover of fibrillar collagen. Enzymes with an activity against the telopeptides have been demonstrated, however their pH optima or cellular origins may preclude a role in the extracellular matrix under normal physiological conditions. This study was initiated to determine if an enzyme with activity at neutral pH against collagen telopeptides is produced by connective tissues.

Medium conditioned by the culture of porcine gingival explants was shown to contain, in addition to collagenase (vertebrate-type), proteolytic activity capable of releasing small fragments from the telopeptides of acid soluble Type I collagen in solution at neutral pH. These low molecular weight peptides did not contain hydroxyproline but did contain the amino acid residue that is involved in cross-linking. For the purposes of this investigation this proteolytic activity was called 'telopeptidase'.

An assay for telopeptidase activity was developed using acid soluble fetal bovine skin collagen reduced with NaB^3H_4,

to label the lysinal (the cross-link precursor), which is located in the telopeptides. The product of this reduction is ^{3}H-hydroxynorleucine.

Two fractions with telopeptidase activity from conditioned medium of porcine gingival explant cultures were separated by gel filtration. The telopeptidases of apparent Mr 70,000 (Fraction A) and Mr 35,000 (Fraction B) were demonstrated to have similar properties. The pH optimum of both telopeptidases were 7.5. As well, both were inhibited by EDTA (0.03M) but not by PMSF (0.002M) nor by NEM (0.002M). Treatment of the telopeptidase of Fraction A with trypsin (soluble or immobilized), pHMB and high and low salt concentrations did not result in any reduction in molecular size. The conditioned medium from cultures of human gingival fibroblasts was also demonstrated to contain telopeptidase activity. This proteolytic activity (of unknown molecular size) had similar characteristics (i.e. pH optimum, and susceptibility to inhibitors) to those described for the telopeptidases from cultures of porcine gingiva.

Incubation of soluble Type I collagen with Fraction A telopeptidase (solution contained low levels of collagenase) at 22°C, resulted in the loss of the major portion of the C-terminal telopeptide. The absence of hydroxyproline in the low molecular weight fragments strongly suggests that cleavage(s) had occured in the extra-helical region. There was no apparent effect on the N-terminal region. Incubation of this proteinase solution with fibrillar collagen at 22°C

resulted in solubilization. A significant proportion of the α_1 chains from the solubilized collagen were devoid of virtually all of the C-terminal telopeptide.

Attempts were made to purify the telopeptidase of Fraction B. However no technique could be found which reproducibly separated this activity from collagenase. Analysis of the products of incubation of the various collagen substrates with Fraction B gave ambiguous results. However, incubation with the radiolabelled collagen resulted in the release of low molecular weight fragments containing ^{3}H-hydroxynorleucine but no hydroxyproline, thereby suggesting that the enzyme had acted within the N- or C-terminal telopeptide.

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Abbreviations

CNBr = cyanogen bromide

DMEM = Dulbecco's modified Eagle medium

dpm = disintegrations per minute

EDTA = ethylenediaminetetraacetate-disodium

pHMB = para-hydroxymercuribenzoate

Mr = relative mass

NEM = N-ethylmaleimide

PMSF = phenylmethylsulphonyl fluoride

SDS = sodium dodecyl sulphate

TCA = trichloroacetic acid

TPCK = L-(tosylamido 2 phenyl) ethyl chloromethyl ketone

TLCK = Nα-tosyl-L-lysine chloromethyl ketone

Vo = void volume (of column)

Vt = total column volume

Standard assay buffer: 0.05M-Tris/HCl containing 0.2M-NaCl, 0.005M-$CaCl_2$, 0.02% (w/v) sodium azide, pH 7.4.

Gel Filtration Chromatography Buffer: 0.05M-Tris/HCl containing 0.2M-NaCl, 0.005M-$CaCl_2$, 0.05% (w/v) Brij®35, 0.03% (v/v) toluene, pH 7.4.

I. INTRODUCTION

A. Extracellular Matrix

Connective tissues are comprised of a variety of cell types which for the most part are surrounded by their specific products. This extracellular matrix is a complex of many types of macromolecules which form the basis of the structural and functional integrity of connective tissues. These components are not static, they are synthesized, modified and degraded, at different rates in the various connective tissues throughout life. The types of macromolecules and their interactions are responsible for the ultimate characteristics of the individual connective tissues.

B. Basic Structure of Gingiva

Histologically, attached gingiva is in many respects similar to other soft connective tissues (Fig 1). It is covered by gingival epithelium whose outer layer is composed of flattened dead cells that are keratinized (Scott and Symons, 1974). The connective tissue or lamina propria (corium) is separated from the epithelium by a basement membrane. The lamina propria is well innervated and has a rich blood supply. The connective tissue supporting the gingival epithelium when in a healthy state, contains many collagen fibres and fibroblasts with no neutrophils being apparent in the extravascular space. The lamina propria

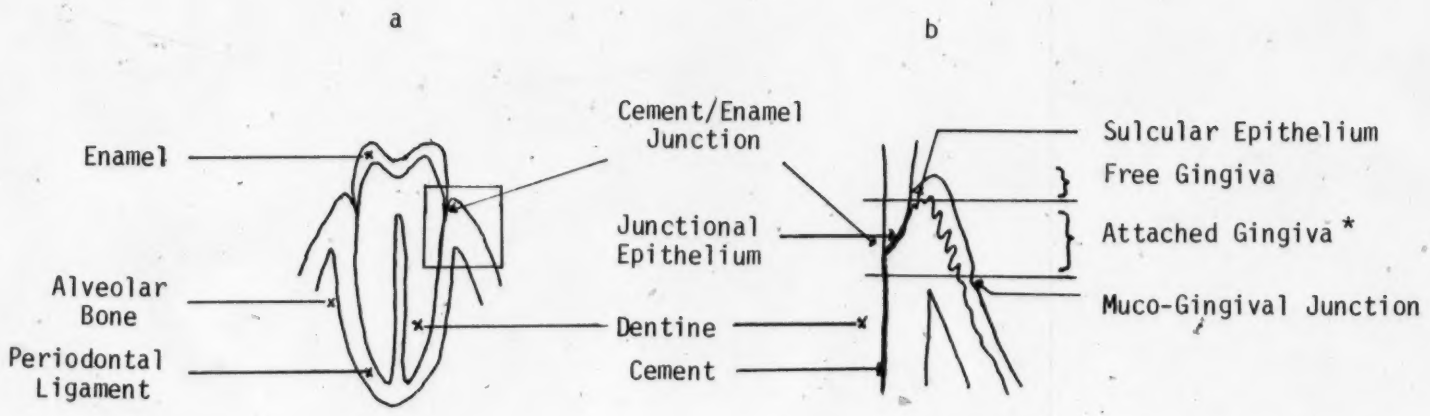


Figure 1. Location of Attached Gingiva.

a) Bucco-lingual section of lower molar and alveolar bone.
b) High magnification to illustrate attached gingiva.

adjacent to the sulcular and junctional epithelium contains by contrast few collagen fibres, but many neutrophils (Osborn and Ten Cate, 1983).

C. Collagen

Collagen Types

Collagen, of which there are several genetically distinct types (reviewed by Gay and Miller, 1983), forms the structural framework of the connective tissues. The collagen types have several common features, one of which is the presence of glycine as every third amino acid residue in the helical region and the large numbers of tripeptides containing the sequence of Gly-Pro-Y or Gly-X-Hyp (Bornstein and Traub, 1979). The sequence, Gly-Pro-Hyp, makes up 10% of tripeptides in the collagen helix (Nimni, 1983). The presence of glycine in every third position enables three collagen chains to associate to form the triple helix. In this conformation, the collagen molecule possesses a high degree of structural integrity, as well as resistance to the majority of non-specific proteinases. Type IV collagen differs from Types I, II and III, in that there are regions within the helix that are not made up of the repeating Gly-X-Y tripeptides (see above), and these regions are susceptible to the action of certain proteinases (Bornstein and Sage, 1980). Type I collagen is composed of 2 α_1 chains and 1 α_2 chain ($[\alpha_1(I)]_2\alpha_2(I)$). Type II and III collagens

are composed of 3 identical chains denoted by $[\alpha_1(II)]_3$, and $[\alpha_1(III)]_3$ respectively. The predominant molecular species for Type IV collagen is unknown (Gay and Miller, 1983). Type I collagen is found in essentially all soft-connective tissues (skin, tendon, ligament) as the predominant collagen as well as in calcified tissues (bone, dentine, and cementum). Type II is found primarily in cartilaginous tissue. Type III is found mainly in soft-connective tissues, however in most tissues other then fetal, it is present at a much lower concentration than that of Type I. Type IV collagen is found only in basement membranes. In addition there are other types of collagen which have been recently described (reviewed by Gay and Miller, 1983).

Biosynthesis of Collagen

Collagen is synthesized by several cell types in various connective tissues in a manner that is similar to other proteins destined for export (reviewed by Prockop, 1982). A collagen gene ($\alpha_2(I)$), has recently been isolated and regions of this gene characterized (Wozney et al., 1981a,b; Crombrugghe and Pastan, 1982). There are approximately 54 coding sequences (exons), ranging in size between 18-108 base pairs. These are separated by intervening sequences (introns). The total length of the transcribed product (mRNA) of this gene is 10 times the length of the functional mRNA that is ultimately translated into the precursor of the $\alpha_2(I)$ chain (see below; Nimni,

1983).

The shortened collagen mRNA is translated and the product, which is called the pre-procollagen, loses it's signal peptide as it enters the endoplasmic reticulum (Nimni, 1983). The individual collagen chains are modified by the action of prolyl and lysyl hydroxylases, which act on certain prolyl and lysyl residues. The extent of hydroxylation, which occurs on α chains in the non-helical conformation, appears to be dependent upon specific factors which are likely to be variable in different tissues (for example substrate concentration and availability; Nimni, 1983). The procollagen chains are further modified by the addition of sugars (galactose and glucose on hydroxylysyl residues within the helix) in the cisternae of the rough endoplasmic reticulum (reviewed by Kivirikko and Myllyla, 1979). Triple helix formation occurs in the endoplasmic reticulum. After the addition of oligosaccharides (containing N-acetyl glucosamine and mannose) to the extension peptides, which probably mainly occurs in the Golgi apparatus, the procollagen is packaged into secretory vesicles, transported to the plasma membrane and secreted from the cells (Nimni, 1983).

The Type I procollagen molecule consists of three polypeptide chains ($[\text{pro-}\alpha_1(\text{I})]_2$ $\text{pro-}\alpha_2(\text{I})$) each made up of 1,014 amino acid residues which form the triple helix, and extension peptides of approximate molecular weights of 20,000, and 35,000 at the amino- and carboxy-termini,

respectively (Bornstein and Traub, 1979). The terminal extensions are believed to prevent premature precipitation of the collagen molecules (collagen fibril formation) within the cell, or before the collagen molecule is actually in place on the growing fibril (Light and Bailey, 1980). The major portion of each extension is removed in the extracellular matrix by the respective N and C-terminal pro-peptidases, at some point up to and including the incorporation of the collagen monomers into new or pre-existing collagen bundles known as fibrils and fibers. The remaining segments of the extension peptides, which are referred to as the telopeptides (16 amino acid residues at the N-terminus, and 25 amino acid residues at the C-terminus), are apparently essential for the proper alignment of the collagen molecules. Gelman et al. (1979) demonstrated that collagen molecules lacking most of the telopeptide regions (removed by pepsin treatment) formed abnormal fibrils lacking the characteristic banding patterns. In addition, the rate of fibril formation was significantly slower when compared to normal collagen. This latter finding was confirmed by Brennan and Davison (1981).

The last major enzyme-catalyzed modification of the collagen, is the conversion of some of the lysyl and hydroxylysyl residues on both telopeptide regions to an aldehyde form by the action of lysyl oxidase. This conversion is a pre-requisite for the formation of stable intra- and intermolecular cross-links, as discussed below.

Covalent Interchain Cross-links in Collagen

The formation of a covalent interaction between collagen chains or molecules is preceeded by the conversion of the lysyl residues of the telopeptide regions (N-9 and C-17 of the α_1 chain and N-5 of the α_2 chain; see Fig 2A), to α-aminoadipic (δ)-semialdehyde, otherwise known as 'lysinal' (the aldehyde derivative of lysine; also referred to as allysine) or the conversion of hydroxylysine to 'hydroxylysinal' (hydroxyallysine; Bornstein and Traub, 1979). Lysyl oxidase which catalyzes this reaction appears to function preferentially on collagen aggregates or fibrils (Siegel, 1974). In addition, the enzyme also appears to prefer hydroxylysine as its substrate.

Several types of interaction have been demonstrated or postulated for these modified lysines. The two main types which have been characterized after reduction with sodium borohydride (thus called the reducible cross-links), are the aldol condensation products and the aldimine cross-links. The former type involves the condensation between two adjacent lysinal residues, while the aldimine cross-links are derived from the condensation of a lysinal (hydroxylysinal) and an epsilon amino group on lysine or hydroxylysine (Bornstein and Traub, 1979; Light and Bailey, 1980).

It is generally accepted that lysyl oxidase acts preferentially on lysyl/hydroxylysyl residues situated at the extra-helical regions of collagen (Bornstein and Traub, 1973). In soft tissue collagen, aldol condensation products

Figure 2. Structure of Bovine Type I Collagen.

A) Type I bovine skin collagen is composed of $2\alpha_1$ and one α_2 chains. Each chain consists of 1,014 amino acid residues which make up the helical regions (represented by the horizontal line), and an additional 16 and 25 residues for the α_1 chain, located at the N and C-terminal extra-helical regions (telopeptides) respectively, and 9 and 6 residues for the α_2 chain respectively. The locations of the methionine residues are denoted by the short vertical lines.

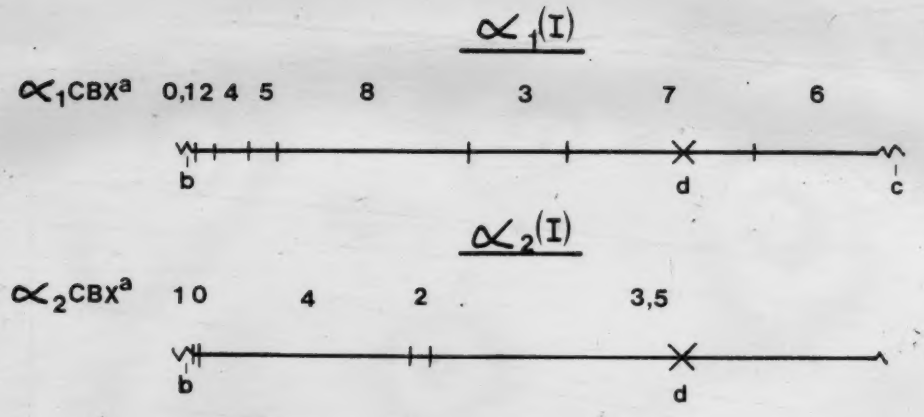
a - denotes the peptides (CBX) derived from the treatment of the collagen with cyanogen bromide.
b - location of the cross-link precursor (N-9) in the N-terminal telopeptide.
c - location of the cross-link precursor (C-17) in the C-terminal telopeptide.
d - location of the bond cleaved by vertebrate collagenase (between residues 775-776).

B) A 2 dimensional representation of fibrillar collagen, showing the 4D stagger (see text) and intermolecular cross-linking between collagen molecules (see text).

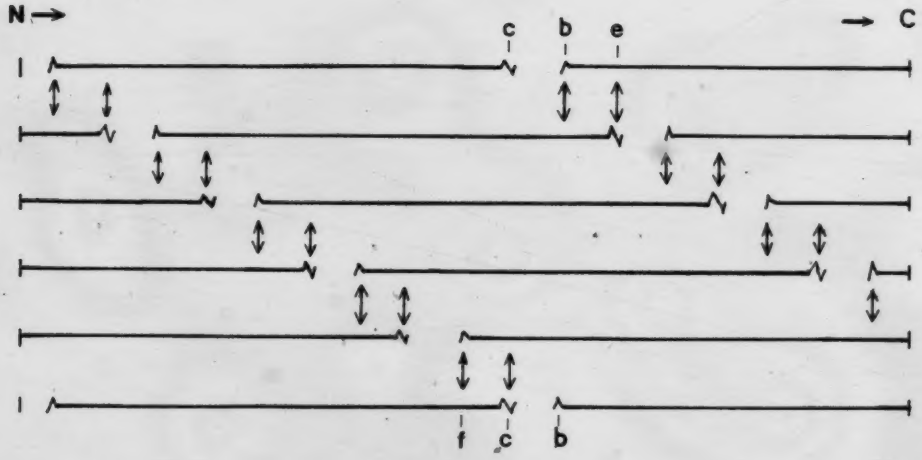
b - residue N-9
c - residue C-17
e - residue 87
f - residue 930

Intermolecular cross-links shown are between residues N-9 and 930, and residues C-17 and 87.

A



B



have been ascribed, in most part, to the N-terminal telopeptide, between adjacent aldehydes N-9 to N-9 of two α_1(I) chains, or an α_1(I) and an α_2(I) (N-5) chains. These interactions are likely to be responsible for all of the intramolecular cross-links found in soft tissue collagens. However there are no known functions for this type of cross-link. Aldimine type cross-links have been demonstrated to involve lysinal in the C-terminal telopeptide (C-17) of one collagen molecule and a hydroxylysine within the helical portion of another collagen molecule (residue 87, in the α_1CB5 peptide; Fig 2B), thus forming an intermolecular cross-link (dehydro-hydroxylysinonorleucine). The location of the hydroxyl group may be reversed in the above two participants in this cross-link, however a similar end product is obtained (Bailey and Peach, 1968). This latter type of interaction (involving hydroxylysinal and lysine or hydroxylysine) undergoes a spontaneous Amadori rearrangement to form a keto-amine, which is chemically more stable, however the physiological significance of this extra stability is not known. Reduction of both dehydro-hydroxylysinonorleucine and the keto-amine with ^{3}H-sodium borohydride yields ^{3}H-hydroxylysinonorleucine, with the only difference being in the location of the tritium (Bornstein and Traub, 1979).

In the soluble collagen from connective tissues such as bovine skin, the predominant cross-link precursor is lysinal (after reduction it is hydroxynorleucine; this is discussed

in more detail in the Results; Fig 10). In insoluble fibrillar collagen from skin the predominant reduced cross-link is hydroxylysinonorleucine, with some lysinonorleucine which is derived from condensation of a lysinal and a lysyl residue (Bornstein and Traub, 1979). Other interactions have been proposed such as an aldol condensation product of two adjacent lysinals (C-17) from the C-terminal extra-helical region (Brennan and Davison, 1981; Davison and Brennan, 1982). However this may be an artefact caused by the extraction techniques or during the reduction with sodium borohydride under basic conditions (personal communication, P.G. Scott).

The initially-formed adducts are considered by some workers to undergo further reaction. The addition of an adjacent histidine to an aldol condensation product would form aldol-histidine as proposed by Tanzer et al. (1973). This in turn may interact with another hydroxylysine to form histidino-dehydrohydroxymerodesmosine. However, as demonstrated by Robins and Bailey (1973), the formation of this reduced component is probably a borohydride-induced artefact. There is no direct evidence for the involvement of histidine in the reducible cross-links of collagen *in vivo* (Bornstein and Traub, 1979).

The cross-links that have been defined above are the so-called reducible cross-links. The levels of these types of cross-links appear to decrease with ageing or maturity of the tissue/organism (Robins et al., 1973). To maintain the

mechanical integrity of the collagen, it would appear that other types of cross-links would be necessary. These have been called the non-reducible (stable, mature) cross-links. Fujimoto and Moriguchi (1978) isolated from bovine achilles tendon collagen, a fluorescent compound which they named pyridinoline. The authors believed that this compound, which was isolated after acid hydrolysis and ion-exchange chromatography, was due to the interaction of a reducible cross-link (between 2 α chains) and a hydroxylysine from a third chain. Pyridinoline was also demonstrated in collagen of rat costal cartilage, rib and femoral bone (Fujimoto and Moriguchi (1978). The content of pyridinoline in the rat skin however was extremely low. Robins (1983) confirmed the existence of pyridinoline in his investigations of sheep long bone collagen, and demonstrated that it probably exists *in vivo* as a galactosylpyridinoline. This cross-link component was also demonstrated in Type II collagen from bovine articular cartilage (Robins and Duncan, 1983). The analysis of certain peptides isolated after cyanogen bromide digestion of the cartilage collagen confirmed that the pyridinoline was involved in cross-linking of three peptides from different chains. According to the above investigators the function of pyridinoline appeared to be in stabilizing the 4D stagger of adjacent collagen molecules. There are other postulated non-reducible cross-links (gem-diamine; Davis et al., 1975; hydroxy-aldol histidine; Housley et al., 1975), however their existence *in vivo* has not been

confirmed (Bornstein and Traub, 1979; Light and Bailey, 1980). As noted by Light and Bailey (1980), to verify that a particular cross-link does exist would require independent analysis of the structure as well as the chemical synthesis of the compound.

Rauterberg (1973) and Zimmermann et al. (1973) described the presence of intermolecular cross-links involving the C-terminal telopeptide region. Comparisons were made by these authors between cyanogen bromide digests of fibrillar collagen from a variety of tissues including bovine skin, and the cyanogen bromide digests of acid soluble collagen. They demonstrated decreased levels of non-cross-linked α_1CB6 peptides from the acid insoluble collagen preparation. With a monospecific antibody directed towards the C-terminal telopeptide, Zimmermann et al. (1973) demonstrated the presence of α_1CB6 in higher molecular weight fractions. Treatment of the fibrillar collagen with pepsin (which removes a major portion of the telopeptides), followed by digestion with cyanogen bromide resulted in the apparent loss of all antigenic determinant that was initially observed in the various fractions, with a concominant rise in free α_1CB6. Intermolecular cross-linking involving the α_1CB6 peptide has been shown to involve 2 different specific interactions (Bornstein and Traub, 1979). The above authors (Zimmermann et al., 1973; and Rauterberg, 1973) believed that some of the intermolecular cross-links were due to the interaction of the C-terminal telopeptide of

one α chain and some region within the helix of a second chain. It has since been described that the latter cross-link joins residue C-17 to residue 87 of α_1CB5 (Fig 2B). Kang (1972) demonstrated in rat tail tendon collagen, the condensation of lysinal from the N-terminal telopeptide of an α_1 chain (α_1CB0,1) and α_1CB6. This cross-link involves residue N-9 and residue 930 of the α_1CB6 respectively (Fig 2B; Bornstein and Traub, 1979). Scott and Veis (1976b) analyzed acid insoluble bovine skin and dentine collagen after digestion with cyanogen bromide. They calculated that, on average, 1.5 cross-links per collagen molecule involved the α_1CB6 peptide. In a later study on highly cross-linked insoluble bovine dentine collagen, Scott and Edwards (1981) calculated the number of intermolecular cross-links that could be attributed to each telopeptide region. On average, there is one cross-link per molecule originating from the C-terminal telopeptide as well as one from the N-terminal telopeptide. In soft tissue fibrillar collagen the exact contribution of each form of interaction appears not to have been deduced.

Collagen Fibre Structure

The intermolecular cross-links appear to arise in the location described above as a result of the way the collagen molecules are organized in the fibrils. However these cross-links are also responsible for stabilizing these fibrils. The collagen molecules are axially displaced by an

integral multiple of D (Miller, 1976). The length of the collagen molecule is 4.4D (280nm), with each D period extending over approximately 234 residues (67nm). This highly ordered array of collagen molecules is depicted in a simplified two-dimensional view in Fig 2B (Hodge, 1967; Bornstein and Traub, 1979). This model is based on the Hodge and Petruska model as discussed in the above references. However this model can only explain the collagen packing in two dimensions (Miller, 1976). Various models have been proposed to account for the organization in three dimensions while keeping the stagger depicted in Fig 2B. One concept, as noted in the review by Bornstein and Traub (1979), involves collagen fibrils which are built up with micro-fibrillar units which are in turn composed of collagen molecules in staggered array. Different investigators have suggested collagen microfibrils are composed in cross-section of 2, 4, 5 or 8 collagen monomers. Another proposal, which is called the quasi-hexagonal packing model, involves collagen molecules packed in an approximately hexagonal lattice but without a microfibrillar substructure (Hulmes and Miller, 1979; Hulmes and Miller, 1981). This organization would essentially be continuous throughout the fibril.

Calf skin collagen fibrils are packed parallel to one another into coarse fibres, which form a mechanically stable network (Kuhn and Glanville, 1980). The sizes of these fibrils vary in different tissues, and may range from

10-100nm diameter (Kuhn and Glanville, 1980). Bone tissue contains thinner fibrils than calf skin. In addition the fibrils of bone have a different appearance on electron microscopy (more of an irregular arrangement). The types of packing and the interactions of the collagen between itself and other extracellular macromolecules are factors which could contribute to the differences in the structures of various connective tissues, and these in turn are presumably related to differences in function.

D. Proteoglycans and their Interactions with Collagen

Proteoglycans are macromolecules consisting of a protein core with one or more long side chains called glycosaminoglycans which are made up of repeating disaccharides. The repeating disaccharide unit consists of hexosamine and hexuronic acid except in keratan sulphate which is composed of repeating units of hexosamine and galactose. In addition, there are short branched chains of oligosaccharides (not containing hexuronate) linked to the protein core. The types and concentration of the proteoglycans are quite variable between the different connective tissues (Chakrabarti and Park, 1980). These macromolecules are in low concentrations except in cartilaginous tissues but may have an important influence on the degree of hydration and osmotic swelling of the connective tissues (Pearson, 1982). Other properties attributed to the proteoglycans are viscoelasticity, modulation of access of

materials to and from the cells and inhibition of calcification (Pearson, 1982).

In soft connective tissues sulphated galactosaminoglycans and hyaluronic acid (which is not covalently linked to protein) are the predominant glycosaminoglycans. Heparan sulphate (a sulphated glucosaminoglycan) though found in lower concentrations, is present in proteoglycans associated with basement membranes and the external surface of cells (Chakrabarti and Park, 1980). One form of heparan sulphate proteoglycan is intercalated with cell membranes (Oldberg et al., 1979). The sulphated galactosaminoglycans of non-cartilage connective tissue are of at least three types; chondroitin sulphate (a homopolymer containing D-glucuronate but no L-iduronate) and two categories of hybrids (or copolymers) that differ in the proportions of L-iduronate and D-glucuronate in their repeating disaccharide units. The L-iduronate-rich hybrid is widely distributed, and is present in bovine periodontal ligament and skin (Pearson and Gibson, 1982; Gibson and Pearson, 1982) and in bovine sclera (Coster and Fransson, 1981). The low iduronate-sulphated galactosaminoglycan has been demonstrated in the same tissues. However in mature skin, 97% of the sulphated galactosaminoglycans was shown to be the hybrid rich in iduronic acid (Gibson and Pearson, 1982). In addition, the two types of sulphated galactosaminoglycans are present in different proteoglycans (Pearson and Gibson, 1982; Coster and Fransson, 1981). The proteoglycan containing the hybrid

rich in L-iduronate (proteodermatan sulphate) has an apparent molecular weight in the range of 70,000-90,000 (Coster and Fransson, 1981; Damle et al., 1982). On the other hand the size of the proteoglycan containing the hybrids low in iduronate has not yet been determined but it is considerably larger than proteodermatan sulphate (Coster and Fransson, 1981; Pearson and Gibson, 1982).

There have been a number of studies on the interactions of proteoglycans and/or glycosaminoglycans with collagen (review: Chakrabarti and Park, 1980). Obrink (1973) demonstrated that the glycosaminoglycans that were called chondroitin-4-sulphate, dermatan sulphate (rich in L-iduronate), heparan sulphate and heparin as well as chondroitin sulphate proteoglycan and proteodermatan sulphate, all bind to lathyritic collagen. Binding appeared to be due to the negative charge. Keratan sulphate and hyaluronic acid did not bind, or were bound weakly. Dermatan sulphate (rich in L-iduronate) appeared to bind most strongly. Various glycosaminoglycans were demonstrated to affect the stuctural stability of collagen in solution (Gelman and Blackwell, 1974). These authors demonstrated that the melting temperature of collagen was increased from 38°C to 46°C after addition of chondroitin-4-sulphate, dermatan sulphate, hyaluronic acid or keratan sulphate.

The close interaction of dermatan sulphate (L-iduronic acid rich) and collagen in skin was demonstrated by the difficulty of extraction of dermatan sulphate from insoluble

collagen other than by techniques that denatured or degraded the collagen (hot 6M-urea, proteolytic digestion; Toole and Lowther, 1966). Laurent (1977) in his review of the interactions of proteins and glycosaminoglycans, discussed the strong electrostatic interactions that do exist between collagen and dermatan sulphate (L-iduronate rich), which may explain the findings of Toole and Lowther (1966). However, according to Laurent (1977), there must also exist a certain degree of specificity in terms of this binding. Chondroitin sulphate and dermatan sulphate (low and high iduronate hybrids) have the same charge density, however, as was shown by Obrink (1973), high iduronate-dermatan sulphate was bound more strongly to collagen. The only difference is the presence of a relatively large amount of L-iduronic acid. Several investigators have described or discussed the effect of high iduronate-dermatan sulphate on collagen fibrillogenesis (Obrink, 1973; Obrink and Sundelof, 1973; Gelman and Blackwell, 1974). Vogel and Heinegard (1983) demonstrated the direct inhibition of fibrillogenesis of acid soluble tendon collagen by the addition of tendon proteodermatan sulphate (Mr 90,000). This effect was not observed with a larger proteoglycan isolated from the same tissue (which contained little or no L-iduronate) or with either large or small proteoglycans of bovine cartilage. The effect was to delay fibril formation, as well as to reduce the overall yield of fibrils (20% of the control at 5 hours).

Electron microscopy of rat tail tendon and rabbit achilles tendon using fairly specific stains for the glycosaminoglycans demonstrated a regular repeating pattern on the collagen fibrils (Scott,J.E., 1980). Scott believed that the glycosaminoglycan seen was the dermatan sulphate component of proteodermatan sulphate. This was based on the known concentration of the glycosaminoglycan *in situ* and the ratios of uronic acid to hydroxyproline. The proteoglycan appeared to be separated by intervals of 65nm on the outside of the fibres, which corresponds to the D-stagger of collagen. Scott and Orford (1981) demonstrated that the proteoglycan filaments were orthogonally arrayed around the collagen fibrils, and that they appeared to be located near the gap region of collagen. No staining for proteoglycan was observed within the fibrils.

The stains used by J.E. Scott cannot visualize the protein core of a proteoglycan directly. However, in this department electron microscopy after staining with specific monoclonal antibodies to the protein core of proteodermatan sulphate (Pringle et al., in press), suggests that the protein core itself is adjacent to the gap region. The presence of proteodermatan sulphate at the gap region might imply that the role is to limit radial growth of the collagen fibrils.

In cultures of actively growing human skin fibroblasts grown on collagen gels, the two forms of proteoglycans (containing low or high iduronate-sulphated

galactosaminoglycans) were produced (Gallagher et al., 1983). The proteodermatan sulphate was found predominantly bound to the collagen gel while almost all of the proteoglycan containing low iduronate-hybrids was in the culture medium. These results taken together with the findings of Scott and of Pringle et al. (see above) strongly suggest a close relationship of collagen and proteodermatan sulphate *in vivo*.

E. Fibronectin and Collagen Interactions

There are a variety of glycoproteins in soft connective tissues (Pearson, 1982). Some of these have been investigated for their effects on collagen fibrillogenesis. Fibronectin is a cell surface and plasma glycoprotein that apparently mediates adhesion of cells to the extracellular matrix (Ruoslahti et al., 1981). It is composed of two chains each of approximately 220,000 daltons, that are joined by disulphide bonds. It has distinct binding regions each being capable of binding certain macromolecules or cells.

Both native and denatured collagen bind to fibronectin (Engvall and Ruoslahti, 1977), however gelatin binds much more strongly in *in vitro* experiments (Jilek and Hormann, 1978). In addition, native Type III collagen had a significantly greater affinity for fibronectin than Type I and II collagens (Engvall et al., 1978). The region in collagen which has the highest affinity towards fibronectin

contains the peptide bonds that are cleaved by vertebrate collagenase (Kleinman et al., 1978). It has been proposed that one of the functions of fibronectin is to serve as a building block for the assembly of the extracellular matrix. This is based on evidence in cell cultures of the incorporation of plasma fibronectin into the matrix (Hayman and Ruoslahti, 1979). Addition of fibronectin to native collagen undergoing fibril formation delayed the precipitation of the collagen while not altering the total amount of collagen fibrils (Kleinman et al., 1981). These authors proposed that fibronectin may regulate the thickness of the collagen fibers.

The interaction of collagen and fibronectin was examined using antibodies specific for the collagen-binding region on fibronectin (McDonald et al., 1982). In cultures of human diploid cells, the addition of these antibodies did not alter the overall production of collagen and fibronectin, however the deposition of the extracellular collagen and fibronectin into a fibril network was prevented. These authors proposed that the interaction of collagen and fibronectin is a prerequisite for normal collagen architecture in cell cultures.

F. Connective Tissue Neutral Proteinases

Collagenase

The classic collagenolytic enzyme, collagenase, has been the focus of a large number of investigations from the time it was first demonstrated in primary cultures of tadpole tail tissues by Gross and Lapiere in 1962. Collagenases have been demonstrated in nearly every connective tissue undergoing collagen degradation (Harris and Cartwright, 1977). As well, collagenases have been demonstrated to be secreted by fibroblasts (Werb and Burleigh, 1974; Stricklin et al., 1977), polymorphonuclear leukocytes (Robertson et al., 1972a,b; Murphy et al., 1977), macrophages (Werb and Gordon, 1975a), and from proliferating rabbit epithelium in culture (Donoff et al., 1971).

The vertebrate collagenases are metalloproteinases requiring both zinc and calcium ions for maximal activity. Zinc appears to be an integral component of the collagenase and cannot be removed without destroying the activity (Seltzer et al., 1977). Calcium, which is an extrinsic requirement for the activity, appears to function as an enzyme activator and as a stabilizer of the tertiary structure of the enzyme at physiological temperatures (Seltzer et al., 1976).

The mechanism of action of vertebrate collagenase from various tissues appears to be similar, at least in terms of the site of cleavage on the collagen molecule. However,

collagenases derived from different sources appear to differ in a few characteristics which are discussed below (Gross, 1976).

A generally accepted definition of collagenase is: a proteinase active at neutral pH and capable of cleaving native collagen in solution into 2 fragments; TC-3/4, which comprises 75% of the collagen molecule containing the N-terminus, and TC-1/4 which makes up the remaining 25% of the molecule including the C-terminus. At physiological temperatures these products denature (for Type I collagen the products are: $\alpha_1$3/4 and $\alpha_1$1/4, and $\alpha_2$3/4 and $\alpha_2$1/4; in addition due to intramolecular cross-links (aldol condensation products originating in the N-terminal telopeptide), there will also be β3/4 components) and thus become susceptible to attack by other proteinases. The melting temperature of the TC-3/4 fragment is 32°C, while that of the TC-1/4 is 29°C (Sakai and Gross, 1967).

The collagenase scissile bonds in different types of collagen have been demonstrated. In the α_1 chain as well as in the cyanogen bromide-derived α_1CB7 (of Type I collagen) from rat and chick skin the scissile bond is between glycine 775 and isoleucine 776 (Gly-Ile-Ala-Gly-Gln-Arg) using collagenase isolated from rabbit tumors (V_2 ascites cell carcinoma) (Gross et al., 1974). In the α_2(I) collagen it is believed to be at the same locus as in the α_1(I) but between glycine and leucine (Gross et al., 1974). The scissile bond in α_1(II) (using collagenase purified from cultures of

rabbit tumors) is between a glycine and isoleucine (Gly-Ile-Ala-Gly-Gln-Arg), and for α_1(III), it is between glycine and leucine (Gly-Leu-Ala-Gly-Leu-Arg) (Miller et al., 1976b). According to Miller et al. (1976b), the minimum sequence required for collagenase cleavage of collagen appears to be Gly-Ile-Ala, or Gly-Leu-Ala. There are other regions on the α chains that have the above or similar sequences, which apparently are not cleaved by the rabbit tumor collagenase. The proximity of hydroxyproline to the potential cleavage sites may be the reason why collagenase is ineffective, according to Miller and co-workers.

There have been reports by some investigators claiming that purified collagenase can cleave native collagen at other loci (reviewed by Harris and Cartwright, 1977). However, according to these authors, any extra cleavages of the collagen may be due to other contaminating proteinases. A collagenase derived from cultures of human gastric mucosa apparently cleaves native collagen at a site that is 77% of the distance from the N-terminal end of the collagen molecule, as determined by examination of segment long spacing crystallites by electron microscopy (Woolley et al., 1976b). This collagenase was demonstrated by these authors to be similar in other respects to other vertebrate collagenases.

It has been shown that human skin collagenase can cleave Types I, II, and III collagen but cannot degrade either Type IV or Type V (Liotta et al., 1979). Liotta et

al. (1981b) demonstrated that a collagenase isolated from a metastatic tumor (Murine) was capable of cleaving Type IV collagen without significantly digesting other collagen types. This collagenase was shown to have similar properties to other known vertebrate collagenases, with a single locus of digestion within the triple helical body of the collagen molecule. A Type V metal-dependent collagenase was demonstrated in cultures of rabbit pulmonary alveolar macrophages (Mainardi et al., 1980b) and from cultures of certain tumor cells (Liotta et al., 1981a).

Purified collagenase acts most rapidly on native collagen molecules. McCroskery et al. (1973), using a collagenase that was isolated from homogenates of V_2 ascites cell carcinoma, demonstrated that gelatin (denatured collagen) was cleaved much more slowly than native collagen. Purified collagenase from cultures of human skin fibroblasts was found by Welgus et al. (1982) to degrade denatured collagen (Types I, II, III, IV and V). The first cleavages in Types I, II and III collagen that were observed by polyacrylamide gel electrophoresis were those that resulted in the production of $\alpha 3/4$ and $\alpha 1/4$ peptides. Continued incubation, however resulted in further cleavages with the end result of all of the peptides being of relatively low molecular weight. McCroskery et al. (1975), using collagenase extracted from rabbit tumors, did not demonstrate any further degradation products of $\alpha 3/4$ and $\alpha 1/4$ peptides. This finding was consistent with the

observations of Gross et al. (1974) and Miller et al. (1976b). Harris and Cartwright (1977) suggest that purified tadpole and rabbit tumor collagenases are quite specific for the one locus on collagen, any additional cleavages being due to contaminating proteinases. Nevertheless it is still possible that collagenases isolated from different tissues may have slightly different specificities, as was discussed above (Woolley et al., 1976b; Gross, 1976).

Welgus et al. (1982) sequenced the first 3 amino acid residues of the unfractionated peptides of collagenase-degraded gelatin, and concluded that the only scissile bonds were between glycine and leucine or isoleucine. In addition these investigators demonstrated that α_2 chains were degraded faster than α_1 chains, however, both were degraded at a slower rate than native Type I collagen.

There are a number of investigations that have demonstrated the different rates of digestion of Types I, II and III collagen with collagenase. Soluble Type II collagen is apparently cleaved at 20% of the rate of Type I and III collagen (Harris and Cartwright, 1977). Horwitz et al. (1977) reported that collagenase from human polymorphonuclear leukocytes degraded Type I collagen at 15 times the rate of Type III collagen but the collagenase from rabbit alveolar macrophages digested Types I and III at equal rates. Collagenases, from cultures of rabbit colon wall (Oyamada et al., 1983), and from human skin fibroblast cultures (Welgus et al., 1981a) degraded Type III at a

faster rate than Type I. As described above there are conflicting data. However the efficiency of degradation may depend on the source of the collagen substrate as well as the source of the collagenase (Welgus et al., 1981a; Baici et al., 1982). Welgus and coworkers (1981a) demonstrated that collagenase from cultures of human skin fibroblasts degraded Type I collagen from human skin at a faster rate than Type I collagen isolated from calf, guinea pig and rat skins. However there was essentially no difference in the rate of digestion (appearance of the α3/4 and α1/4 peptides) between the three denatured collagen types (Types I, II and III; Welgus et al., 1981a, 1982).

Reported molecular weights of collagenase isolated from a variety of tissues range from 30,000 to 65,000 daltons. There have also been reports which suggest that collagenase may be found as oligomers. Birkedal-Hansen et al. (1976) concentrated the conditioned medium of rabbit alveolar macrophage cultures, and by gel filtration of the medium (not pre-treated with agents that would activate latent collagenase) obtained apparent Mr's of 45,000, 85,000 and 165,000 for the collagenolytic activities. These authors demonstrated that at low salt concentrations there were reversible changes between the oligomers, while at high salt concentrations (1.0M-NaCl, 1.4M-KCl), the 45,000 dalton collagenase species predominated. Dabbous et al. (1977) also demonstrated oligomeric species of collagenase isolated from VX-2 rabbit carcinoma (the medium containing the

collagenolytic activity was not artificially activated), with the apparent Mr's being multiples of 34,000.

There is some controversy in the literature about the origin of latency of collagenase (Harris and Vater, 1980; Barrett, 1980). Latent collagenase may be produced either as a proenzyme (zymogen) (Stricklin et al., 1977, 1978), or found as an enzyme-inhibitor complex (Shinkai and Nagai, 1977; Sellers et al., 1977). These latter investigators believed that the latency of collagenase isolated from cultures was due to the formation of enzyme-inhibitor complexes because treatment of the latent enzyme with either sodium iodide (chaotropic agent) or 4 amino-phenylmercuric acetate (thiol-blocking agent) caused complete activation. Collagenase inhibitors have been isolated from dog serum (Woolley et al., 1976a), human tendon (Vater et al., 1979b), rabbit bone (Cawston et al., 1981) and other tissues (reviewed by Murphy and Sellers, 1980). Some of these inhibitors may also inhibit other metal-dependent connective tissue neutral proteinases. Sellers et al. (1979) isolated an inhibitor from rabbit bone that blocks the gelatin-degrading and proteoglycan-degrading activities as well as the collagenase activity (proteolytic activities were isolated from cultures of rabbit bone). There are also a number of reports suggesting formation of irreversible complexes between isolated inhibitors and active collagenase (Vater et al., 1979b; Sakamoto et al., 1981; Kerwar et al., 1980).

Stricklin et al. (1977,1978) purified 2 collagenase zymogens from cultures of human skin fibroblasts, with molecular weights of 55,000 and 60,000, as determined by ultracentrifugation and SDS polyacrylamide gel electrophoresis. The active forms (after treatment with trypsin) had molecular weights of 45,000 and 50,000. Amino acid analysis of the zymogen forms demonstrated little difference between the two species. Comparison with the active forms would indicate that a small basic peptide was lost on activation. There was, apparently, no hexose nor any amino sugars (glucosamine and galactosamine) associated with any of the forms of collagenase. Cyanogen bromide peptides of the two zymogens were compared. Eight of the nine cyanogen bromide peptides from both species co-migrated on electrophoresis, with only one peptide from each being different. It was also demonstrated by this group that procollagenase may undergo autoactivation without concominant reduction in molecular weight. This suggests that a conformational change was all that was required to expose the active site. Tyree et al. (1981) isolated a collagenase activator from cultures of human skin and rat uterus of an apparent Mr of 110,000. Activation occurred without a demonstrated reduction in size of the latent collagenase.

Procollagenase has been isolated from cultures of rat uterus and analyzed (Roswit et al., 1983). Two sets of zymogens and their corresponding active forms were

demonstrated, with similar molecular weights as was described by Stricklin et al. (1977, 1978) for the collagenase isolated from human skin fibroblast cultures. Amino acid analysis of these zymogens showed greater proportions of glycine and serine, as well as a higher content of acidic amino acids than in the human skin fibroblast procollagenase.

Nagase et al. (1981) extracted the mRNA from rabbit synovial fibroblasts, and, in an *in vitro* system, demonstrated that collagenase was synthesized as a pre-pro-enzyme of Mr of 59,000. In the presence of the microsomal membrane this precursor was modified into a pro-enzyme form of Mr of 57,000. In a recent report, Nagase et al. (1983) demonstrated by pulse-chase and continuous - labelling experiments, that collagenase was synthesized and secreted from the rabbit synovial cells in 2 forms with an Mr of 57,000 and 61,000. Treatment of the cells in culture with tunicamycin (which blocks glycosylation: N-linked oligosaccharides) resulted in the cells producing only the pro-collagenase species of Mr of 57,000. It is possible that the difference in terms of the absence of hexose as noted by Stricklin et al. (1978; see above) and the findings of Nagase et al. (1983) may represent a species difference for the isolated collagenase.

One of the major controversies in the literature over the turnover of collagen concerns the actual role of collagenase in the degradation of fibrillar collagen.

Leibovich and Weiss (1971) reported that collagenase from human rheumatoid synovium had no effect on insoluble collagen. These investigators isolated fibrillar collagen from normal and rheumatoid human synovium (post mortem 60-75 years of age). The collagen was dispersed by treatment with crude α-amylase or EDTA. This collagen was shown to be resistant to the action of both a crude and partially purified preparation of collagenase from cultures of human synovium, at both 27°C (less than 2% release of collagen in a 40 hour incubation, as determined by hydroxyproline analysis) and at 37°C (less than 5% release). These investigators also demonstrated that acid soluble collagen allowed to form fibrils (reconstituted; soluble collagen which is gelled by incubation at 37°C in neutral buffers), was digested and solubilized by the collagenase, but at a slower rate than if the collagen was still in solution. Harris and Farrell (1972) demonstrated that the introduction of 3 methylene cross-links per molecule of collagen (guinea pig acid soluble collagen that was reconstituted and treated with formaldehyde), decreased the rate of degradation (by rheumatoid synovial collagenase) by a factor of 5-10 fold. Harris and McCroskery (1974) demonstrated the apparent resistance to digestion of cross-linked Type II collagen by the synovial collagenase. Vater et al. (1979a) studied the action of collagenase, isolated from cultures of rheumatoid synovium, on reconstituted lathyritic chick bone collagen fibrils cross-linked *in vitro* as a result of the action of

lysyl oxidase. They demonstrated that the introduction of as few as 0.1 cross-links (aldimine type) per mole of collagen caused a distinct resistance to the collagenase. In addition, Vater et al. (1979a) examined the residue of the *in vitro* cross-linked collagen after a certain proportion of it had been solubilized by the action of collagenase. The residue showed no evidence of the characteristic collagenase-derived $\beta 3/4$ or $\alpha 3/4$ and $\alpha 1/4$ peptides, even though there was a significant solubilization of the collagen. The authors concluded that the collagenase was not acting on the collagen that was cross-linked. However, as noted by Gross (1976), other investigators have demonstrated solubilization of polymeric collagen by preparations of collagenase. Gross (1976) discusses an unpublished investigation in collaboration with Harper in which they incubated semi-purified tadpole tail collagenase with the identical substrate used above by Leibovich and Weiss, under the same conditions, and obtained a 50% solubilization. Woolley et al. (1978) purified collagenase by gel filtration and ion exchange chromatography and demonstrated homogeneity by SDS polyacrylamide gel electrophoresis. Incubation at 37°C, for 24 hours, of 30μg of collagenase with 750μg of polymeric collagen (human skin; isolated with α-amylase) resulted in a release of 38% of the collagen. However Woolley et al. (1978) did note that digestion occurred at a much slower rate (5%) when compared to reconstituted collagen.

Other Connective Tissue Neutral Proteinases

The secretion of other neutral proteinases is associated with the secretion of the collagenases in many different tissue systems. For the most part, the study of the role of these proteolytic activities in the turnover of the extracellular matrix has been quite limited. However the functions that have been ascribed to these neutral proteinases are far-reaching.

Certain neutral metalloproteinases have been shown to degrade the core proteins of the proteoglycans, which in an indirect or direct fashion, could possibly assist in the turnover of collagen by exposing it to other proteinases (Dingle, 1975; see section on proteoglycan and collagen interactions). Sapolsky et al. (1976) extracted a neutral metalloproteinase from articular cartilage which degraded the protein core of cartilage proteoglycan. Malemud et al. (1979) isolated an enzyme from cultures of lapine chondrocytes with activity against proteoglycans, while Huybrechts-Godin and Vaes (1978) described an activity isolated from rabbit skin and synovial fibroblasts in culture that degrades the protein core of cartilage proteoglycans.

Galloway et al. (1983) isolated a metalloproteinase from rabbit bone culture medium, with an apparent Mr of 24,500, capable of degrading the core of cartilage proteoglycans. This enzyme was purified and shown to be capable also of degrading Type IV collagen, Type I procollagen

(removed the extension peptides), Type III collagen helix, gelatin, laminin and fibronectin. The substrate 2,4 dinitrophenyl-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH2, was cleaved between the glycine and isoleucine residues.

Neutral proteinases have been shown to have the ability to activate other proteinases, including collagenase, that are in latent form. Werb et al. (1977) proposed that both a plasminogen activator and a latent collagenase were synthesized and secreted by rheumatoid synovial cells in culture. Addition of plasminogen to the cell cultures resulted in the activation of the collagenase. Woessner (1977) also was able to demonstrate, with a serine neutral proteinase from cultures of involuting rat uterus, the activation of a latent form of collagenase isolated from the same cultures. Rheumatoid synovial fluid (human) has been demonstrated to contain a proteinase that appeared identical to that of plasma kallikrein (Nagase et al., 1982). Addition of this isolated proteinase to a preparation of latent collagenase from pig synovial fluid, caused activation of the collagenase.

Sellers et al. (1978), in an investigation of cultures of rabbit bone explants, were able to separate the latent forms of three distinct metalloproteinase activities that, upon activation, degraded collagen (collagenase), gelatin and proteoglycans respectively. The gelatin degrading proteinase was able to digest Azocoll, but not native collagen, articular proteoglycans or azocasein. The third

neutral proteinase was able to degrade azocasein, Azocoll and the proteoglycans, but did not have any activity towards collagen or gelatin.

Neutral proteinases with activity against gelatin, have been isolated from many tissues (reviewed by Harris and Cartwright, 1977). A neutral endopeptidase capable of degrading gelatin to small fragments was demonstrated in cultures of rheumatoid synovium (Harris and Krane, 1972). This proteinase was shown to have twice the apparent Mr of collagenase isolated from the same source, but with similar inhibitory properties. The enzyme was shown to be active against the synthetic Pz peptide (Pz-L-Pro-Leu-Gly-Pro-D-Arg), cleaving between leucine and glycine. Another proteinase isolated from rabbit serum with an activity towards the Pz-peptide was described by Nagelschmidt et al. (1979). The activity appeared to be due to a serine proteinase, which had an apparent Mr of 124,000. It was also shown to be active against denatured collagen and collagen peptides. A Pz-peptidase activity, of an apparent Mr of 77,000 and a pI of 5.0 that was isolated from chick embryos, was shown to have no detectable activity against collagen, collagen α chains and certain cyanogen bromide cleavage products of native collagen (Morales and Woessner, 1977). However it did cleave certain collagen peptides of 5-30 amino acid residues. This proteolytic activity, which was shown to be completely inhibited by pHMB and partially by NEM and chelating agents, was proposed by

these authors to be involved at a late stage in collagen breakdown.

Neutral Proteinases of Polymorphonuclear Leukocytes and Macrophages

Polymorphonuclear leukocyte elastase and cathepsin G are neutral serine proteinases that have been demonstrated to have a large spectrum of substrates including native soluble and insoluble collagen (Starkey, 1977; Starkey et al., 1977). Incubation of elastase and cathepsin G with articular cartilage resulted in the degradation of the proteoglycans followed by the solubilization of Type II collagen monomers. Elastase, but not cathepsin G, when incubated with insoluble bovine tendon collagen (predominantly Type I), also caused solubilization. This collagen, which was examined by polyacrylamide gel electrophoresis, contained no β or γ components. The leukocyte elastase had a similar effect on acid soluble collagen (Types I and III, from rabbit skin) in that there was an absence of β and γ components and an increase in the α chain concentration. The authors concluded that the elastase was acting on the amino-terminal extra-helical region, containing the inter- and intramolecular cross-linking site. However, additional cleavages of Type I collagen were observed on the polyacrylamide gels. Thus the activity of elastase was not restricted to the amino-terminal extra-helical region but the enzyme was also capable of

acting within the helix. Cathepsin G, pancreatic elastase, trypsin or chymotrypsin had negligible effects on the acid soluble collagen (Type I).

Kobayashi and Nagai (1978) also demonstrated similar effects with the leukocyte elastase on the amino-terminus of acid soluble collagen. In addition, these investigators separated a metal-dependent collagenase (apparent Mr of 75,000) and a metal-dependent proteinase with an activity against gelatin (Mr of 150,000), from the elastase (Mr of 25,000). Recently it was demonstrated that leukocyte elastase was able to release the C-terminal telopeptide containing the cross-linking region (Scott and Pearson, 1983). This region, according to these authors, is potentially more important for the formation of inter-molecular cross-linking in soft tissue collagen than is the N-terminal region.

Gadek et al. (1980) demonstrated that elastase from normal human neutrophils, under conditions in which there were no effects on the helix of Type I collagen, degraded Type III collagen. The product was very similar to those of the characteristic α3/4 and α1/4 peptides produced by collagenase. Mainardi et al. (1980a) reported that the bond cleaved (between Ile-Thr) by leukocyte elastase was 4 amino acid residues on the carboxyl side of the site of vertebrate collagenase cleavage. Trypsin (Miller et al., 1976a) and thermolysin (Wang et al., 1978) are also capable of digesting Type III collagen in the collagenase-sensitive

region, but are without effect on Type I collagen (within the helix).

A neutral proteinase isolated from cultures of rabbit VX-2 carcinoma was shown to cleave within the N-terminal telopeptide of native Type I collagen (Dabbous et al., 1977). This enzyme, with apparent Mr of 18-20,000, was inhibited by soybean trypsin inhibitor and thus it is likely to be a serine proteinase. Whether it is an elastase-like proteinase is not known.

Macrophages in culture have been demonstrated to secrete a variety of neutral proteinases, including collagenase (Werb and Gordon, 1975a) and a metal-dependent elastase (Werb and Gordon, 1975b; Banda and Werb, 1981). The effects of this elastase on collagen have apparently not been studied.

Collagenolysis by Phagocytosis

Investigations of peridontal ligament (porcine and monkey), a tissue with normally high levels of matrix turnover, have not demonstrated the presence of an active collagenase (Pettigrew et al., 1980). However Christner (1980) demonstrated the presence of collagenase cleavage products from human periodontal ligament that was allowed to autolyse for varying times at 25°C. Using electron microscopy of serial sections, it was shown in cultures of periodontal ligament fibroblasts incubated with fibrillar collagen, that ingestion and degradation of the collagen

were occurring (Svoboda et al., 1979). Other investigators have also reported the phagocytosis of fragments of fibrillar collagen in normal tissues (Ten Cate and Syrbu, 1974; Beersten et al., 1978). Phagocytosis of collagen has also been demonstrated with serial sections of human gingival fibroblasts *in vivo* (Melcher and Chan, 1981).

It would seem logical that phagocytosis of large fragments of fibrillar collagen by these cells would involve either membrane bound proteinases, or the secretion of proteinases in the vicinity of the cell membrane-collagen contacts, to enable the collagen fibrils to be cleaved and engulfed. Cathepsins, with activities against collagen have been described, however most of these proteinases are only active at acid pH (Burleigh, 1977). It is possible that at the site of cleavage of the fibrillar collagen, the cell membrane creates a pocket into which the acid hydrolases can be secreted and in which the pH is reduced to a level where these proteinases are active. Cathepsin B, a cysteine proteinase can effectively degrade insoluble Type I collagen at pH 3.6 and 37°C (Burleigh et al., 1974; Etherington, 1977). Cathepsin N (previously known as collagenolytic cathepsin) at pH 3.5 and 28°C, solubilized fibrillar tendon collagen, and also converted the β and γ chains of acid soluble collagen in solution to α chains (Etherington, 1976, 1977). Cathepsin D, an aspartate proteinase, at pH 4.0 and 25°C, can cleave fetal bovine skin acid soluble collagen in the C-terminal extrahelical region, N-terminal to the

cross-linking region (Scott and Pearson, 1978b). Incubation with acid insoluble collagen resulted in the release of collagen monomers, which were demonstrated to be lacking the majority of the C-terminal telopeptide. In a later study, the above authors demonstrated shortening of the C-terminal telopeptide of acid soluble Type I collagen with Cathepsin B (Scott and Pearson, 1983).

Collagen Turnover

Turnover of collagenous tissues must occur during growth and development and in wound-healing (Bornstein and Traub, 1979). While the rate of turnover of collagen in most adult animal tissues is low, a small amount of newly synthesized collagen can be found in tissues such as skin. This synthesis must be balanced by collagen degradation. The turnover of collagen was demonstrated to be significantly greater in oral soft tissues, than in skin (Sodek, 1977). This investigator examined collagen synthesis and incorporation into mature collagen, in a number of connective tissues, using ^{3}H-proline (and conversion into ^{3}H-hydroxyproline) as a marker. Both the rate of synthesis and incorporation of newly synthesized collagen into fibrils was highest in periodontal ligament, followed by attached gingiva and then skin. Sodek (1977) calculated that the half life of the mature collagen in periodontal ligament was only 1 day, as opposed to 5 days for attached gingiva, and 15 days for skin.

Collagen turnover is accelerated in pathological conditions and in wound-healing. The increase appears to be related to the infiltration of polymorphonuclear leukocytes and macrophages. Both types of cell have been demonstrated to elaborate collagenase and other neutral proteinases, some of which have been shown to be effective against the telopeptide regions of collagen (as described above). Neutrophil leukocytes are different from other cells that produce collagenase in that these metalloproteinases are stored in the 'specific' granules within the cells (Robertson et al. 1972a,b; Murphy et al., 1977). The polymorphonuclear leukocytes and macrophages, in addition to producing proteinases active against collagen, likely have other roles in the turnover of collagen. Macrophages have been shown to be capable of phagocytosis of collagen (Caputo et al., 1981; Shoshan, 1981). In addition, these cells appear to secrete factors which stimulate fibroblast motility as well as fibroblast proliferation (Leibovich and Ross, 1975,1976), and cause activation of the resident latent proteinases (Horwitz et al., 1976). The latter authors analyzed cultures of alveolar macrophages and found that 2 fractions, with apparent Mr of 20,000 and 11,000, when added back to the conditioned medium, had the ability to double the collagenase activity. Macrophages in culture also apparently secrete a factor which stimulates production of collagenase and neutral proteinase by chondroblasts in culture (Deshmukh-Phadke et al., 1978; Ridge et al., 1980) and by

fibroblasts in culture (Laub et al., 1982).

Wound-healing involves the cooperative effects of various cell types, some of which have been described above. During the initial stages of wound healing, the site is infiltrated with polymorphonuclear leukocytes and mononuclear cells (Ross, 1980). These latter cell types appear to differentiate to become the macrophages. The concentration of the polymorphonuclear leukocytes normally returns to background levels within 1 week. Simpson and Ross (1972) described their investigation in which guinea pigs were made neutropenic with antisera for neutrophils. In the absence of infection wound repair was unaffected. Investigations into the macrophage contribution during wound repair indicated the close interaction of these cells and fibroblasts. Suppression of macrophages by anti-macrophage serum caused a delay in wound repair (Leibovich and Ross, 1975). The macrophage levels at the site of wound repair may remain elevated for several weeks, however, collagen turnover may remain high for several months (Madden, 1977). One possible explanation for this is that the resident fibroblasts which have the capability for producing collagenase and other neutral proteinases in culture, are the main cells that are involved in the degradation of collagen at this later stage.

G. Object

It has been described in this introduction that there is a certain amount of controversy over the initial steps in fibrillar collagen breakdown. The apparent lack or minimal effect of collagenase on insoluble cross-linked collagen, as described by Leibovich and Weiss (1971) and Vater et al. (1979a), has led to the suggestion that there may be a distinct proteolytic activity capable of releasing collagen monomers or aggregates from fibrillar collagen. It has been suggested by Rauterberg (1973) that intermolecular cross-links originating from the amino- and carboxy-terminal telopeptides may modulate the degradation of fibrillar collagen. The removal of these regions containing the cross-links, as proposed by Harris and Cartwright (1977) and others, may be a prerequisite for effective collagen turnover. As described above, there are proteinases known to be capable of removing the telopeptides (e.g. leukocyte elastase and cathepsins). However it is uncertain whether these proteinases do have a role under normal physiological conditions.

This study was initiated to determine the initial steps involved in fibrillar collagen breakdown. The main object of this investigation was to demonstrate whether connective tissues can elaborate a neutral proteinase with a specificity towards the telopeptides. The connective tissue chosen for this investigation was porcine gingiva. Sodek (1977) demonstrated that the rate of collagen turnover in

gingiva was substantially higher than that in skin. If the associated degradation is primarily due to the action of extracellular proteinases, there may be a larger concentration of these enzymes in the medium conditioned by the gingival explants than could be obtained from cultures of other readily available tissues. The conditioned medium from porcine gingival explants has been demonstrated to contain collagenase and other neutral proteinases (Pettigrew et al. 1978, 1981). In addition, this conditioned medium was shown to contain proteolytic activity capable of solubilizing insoluble fibrillar collagen (Pettigrew, 1978; Pettigrew et al., 1978).

If there is a proteolytic activity directed against the telopeptides of collagen ('telopeptidase') then attempts would be made to separate this from the collagenase. In addition, the proteinase would be characterized (molecular weight, inhibitors, pH optimum, scissile bond(s) that is/are cleaved etc.). The roles of these two proteolytic activities, collagenase and 'telopeptidase' (if the activities are indeed due to different proteinases), in the breakdown of fibrillar collagen would then be investigated in the hope that a better understanding would be obtained of this process.

II. MATERIALS AND METHODS

Proteinases, Activators, Inhibitors and Substrates

Cathepsin D was isolated from bovine thymus and purified in Dr. Pearson's laboratory (Scott and Pearson, 1978a). The stock solutions contained 100µg/ml or 1mg/ml of protein in 0.02M-sodium phosphate pH 7, and were stored at -20°C. Antibodies specific for the carboxy-terminal extra-helical region of Type I collagen (residues C-4 to C-9), were prepared by Dr. P.G. Scott (Scott, 1982). Elastase isolated from polymorphonuclear leukocytes was kindly supplied by Dr. A.J. Barrett, Biochemistry Department, Strangeways Research Laboratories, Cambridge, England. Pig synovial collagenase was a gift from Dr. T.E. Cawston, Cell Physiology Department, Strangeways Research Laboratories, Cambridge, England.

TPCK-(L-(tosylamido 2 phenyl) ethyl chloromethyl ketone)-treated trypsin (228 units/mg), pepsin (2,720 units/mg, twice crystallized), and bacterial collagenase (326 units/mg, code-CLSPA) were purchased from Worthington Co. (Freehold, N.J.). Trypsin bound to agarose, soybean trypsin inhibitor, pepstatin, benzamidine hydrochloride, 6-amino hexanoic acid and p-hydroxymercuribenzoate (pHMB) and azocasein were obtained from Sigma Chemical Co. (St. Louis, Mo.). N-ethylmaleimide (NEM), was from Aldrich Chemical Co. (Wisconsin). Phenylmethylsulphonyl fluoride (PMSF) was purchased from Pierce Chemical Co. (Rockford,

Il). Azocoll (50-100 mesh) was obtained from Calbiochem-Behring, (San Diego, Ca.).

Tissue Culture Materials

Heat-inactivated fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), amphotericin B and nystatin were obtained from GIBCO Canada Inc. (Burlington, Ont.). Penicillin, streptomycin, kanamycin, gentamycin, cyclo-heximide and HEPES (N-2-hydroxyethyl piperazine N'-2 ethane-sulfonic acid) were purchased from Sigma. Trypsin 1:250 (Difco certified) and lactalbumin hydrolysate (Bacto-Peptone) were purchased from DIFCO Laboratory (Detroit, Mich.). Concanavalin A was obtained from Pharmacia (Canada) Ltd. (Que.). Blood agar plates were obtained from the Microbiology Department, University of Alberta.

Polyacrylamide Gel Electrophoresis Materials

Polyacrylamide, BIS (N,N' methylene-bis-acrylamide), SDS (sodium dodecyl sulphate), TEMED (N,N,N',N' - tetra-methylethylenediamine) ammonium persulphate, bromophenol blue, Coomassie Blue (R250), Tris (tris (hydroxymethyl) aminomethane) and the mixed bed ion exchange resin AG 501-X8(D) (20-50 mesh) were obtained from Bio-Rad Laboratories (Mississauga, Ont.).

Column Chromatography

Sephadex™ G25 (medium), G100 (fine), G200 (fine), G200 (superfine), DEAE-Sepharose™ CL 6B, cyanogen bromide-activated Sepharose 4B and a protein calibration kit for gel filtration were obtained from Pharmacia. Bio-Gel® P4 (100-200, 200-400 mesh) and P10 (100-200 mesh), Bio-Gel agarose A0.5 (100-200 mesh) and A1.5, (200-400 mesh) were obtained from Bio-Rad Laboratories. Heparin bound to agarose was from Sigma.

Radioisotopes

Tritiated sodium borohydride (110mCi/mMole) and ^{14}C-acetic anhydride (119mCi/mMole) were obtained from Amersham/Searle Co. (Oakville, Ont.). Tritiated water (0.25mCi/g) was purchased from New England Nuclear, Lachine, Que. Scintillation fluid was obtained from various sources as described in Methods.

Other Reagents and Products

Ammonium sulphate (enzyme grade), chloramine T, p-aminobenzaldehyde, hydroxy-L-proline, and bovine serum albumin (radio-immunoassay grade) were from Sigma. Cyanogen bromide was purchased from Aldrich Chemical Co, and stored at -20°C. Gelatin was obtained from J.T. Baker Chemical Co. (Phillipsburg, New Jersey).

Other chemicals and disposables were obtained from Fisher Scientific, Edmonton, and CanLab, Edmonton. All

chemicals utilized in this study were of analytical grade. The water was either double distilled, or singly distilled and further purified through a Milli-Q™ system with a 0.22μm Milli-Stack™ filter (Millipore, Ltd. Mississaugua, Ont.).

A. Tissue Culture

Porcine Gingival Explant Culture

Porcine mandibles (12-18) were obtained within one-half hour of slaughter from a local abattoir (Canada Packers, Edmonton), packed on ice, and processed within 2 hours. The protocol was generally as described by Pettigrew (1978) and Pettigrew et al. (1978). The gingiva was brushed with phosphate buffered saline to remove debris. Attached keratinized gingiva (see Fig 1) from the mandibular molars that was judged to be non-inflamed on visual examination, was excised and placed in Petri dishes with DMEM (pH 7.4) which contained 0.042M-sodium bicarbonate, 100 units/ml penicillin, 100μg/ml streptomycin, 50μg/ml gentamycin, 100μg/ml kanamycin, 3μg/ml nystatin and 3μg/ml amphotericin B. The strips of gingiva were transferred to a sterile 125ml Ehrlenmeyer flask, washed twice with sterile culture medium, and then chopped into 1-2mm^3 sections, with scissors or blades. The pieces were washed extensively with medium, and then distributed into Falcon™ Brand 75cm^2 tissue culture flasks (0.5-0.8g wet weight of tissue per flask).

Medium (13ml) was added and culture was carried out in a humid atmosphere of 5% CO_2 in air at 37°C. Cycloheximide (3µg/ml) was added to some cultures to inhibit *de novo* protein synthesis. The conditioned medium was removed daily, clarified by centrifugation (1000 x g) for 10 minutes and stored in separate tubes at -20°C. Flasks that contained medium which was cloudy were rejected due to the likelihood of bacterial contamination. In addition, a sample from each flask on each day was applied to a blood agar plate, and these were incubated for several days at 37°C. Conditioned medium which showed signs of contamination was discarded, including that from earlier days in culture in these flasks.. Such contamination was found only rarely in this study.

Human Gingival Fibroblast Culture

Normal human gingival fibroblasts (Gin-1, American Type Culture Collection (Md. USA) obtained at the third passage, were initially processed according to the accompanying instructions. The ampule containing the frozen cells was thawed in a 37°C water bath with vigorous shaking (within 30 seconds), and then immersed in 70% (v/v) ethanol, at room temperature. The contents of the ampule were transferred (under sterile conditions) to a 15ml centrifuge tube containing 10ml of DMEM/10% fetal bovine serum with penicillin (100µg/ml), streptomycin (100µg/ml) gentamycin (50µg/ml) and kanamycin (100µg/ml). The fibroblasts were

pelleted, then resuspended in fresh medium, and transferred to a 75cm² Falcon flask, and incubated for 3 days under the same conditions described previously for gingival explants but in the presence of 10% fetal bovine serum.

Cells grown to confluence in the flasks were passaged by the following procedure. The cells were washed three times with phosphate buffered saline, and then once briefly (5 seconds) with a sterile solution of 0.25% (w/v) trypsin (Difco) in phosphate buffered saline. Fresh trypsin solution was added and after 2-4 minutes, the rounded cells were dislodged by repeated aspiration with a Pasteur pipet. The cells were transferred to 15ml centrifuge tubes containing medium with fetal bovine serum and sedimented by centrifugation (1,000 x g for 10 minutes). The cells were resuspended in fresh medium and transferred to new flasks in a ratio of 1:2 or 1:5. The volume was made up to 15ml, and the cells allowed to incubate under the conditions described above. Medium was changed every third or fourth day. Confluence was generally reached after 3-5 days.

After the third passage (sixth passage overall for these fibroblasts) the cells were processed as described by Hurum et al. (1982). The culture medium was DMEM/10% fetal bovine serum, with HEPES (4.7mg/ml), gentamycin (50µg/ml), penicillin (100µg/ml), and amphotericin B (300ng/ml), adjusted to pH 7.2. Once confluent, the cells were washed with phosphate buffered saline and cultured in DMEM without serum but containing concanavalin A (25µg/ml) and

lactalbumin hydrolysate (5mg/ml). The latter product was added to enhance cell viability and to retard cell detachment (Paul, 1975). The conditioned medium was changed daily (4-5 days) clarified by centrifugation and stored at -20°C. The cultures were still viable at end of treatment (Scott et al., 1983).

B. Treatment of Conditioned Medium

Concentration of Conditioned Medium

Two concentration techniques were used in the preparation of neutral proteinases. At all times during the concentration and dialysis procedures, the conditioned medium was kept on ice, or at 4°C. In the earlier stages of the investigation the medium was concentrated (6-60 fold) in a Diaflo™ ultrafiltration apparatus (Amicon Co., Lexington, Mass.) with PM10 membranes (molecular weight cutoff 10,000). At a later stage, concentration of the conditioned medium was accomplished by precipitation by the addition of solid ammonium sulphate (enzyme grade) according to the technique described by Dixon and Webb (1964). This involved the addition of sufficient solid ammonium sulphate to the medium to give 20% saturation. After 2-16 hours the precipitate was removed by centrifugation at 20,000 x g, for 45 minutes. To the supernatant, ammonium sulphate was added in 3-4 aliquots to give 60% saturation. This mixture, which was generally left overnight, was then centrifuged at 30,000 x g for

1 hour. The precipitate was dissolved in a small volume of buffer (0.05M-Tris/HCl, pH 7.4, containing 0.2M-NaCl, 0.005M-$CaCl_2$, and 0.02% (w/v) sodium azide) and thus concentrated 25-50 fold. This buffer was the standard assay buffer used in this investigation. The solution was dialyzed for 48 hours against 2 or 3 changes of assay buffer and then stored frozen at -20°C, until needed.

In the latter stages of this investigation the protocol of Cawston and Tyler (1979) was followed. This is similar to the above procedure except for the following modifications. Conditioned medium that was made to 20% saturation with ammonium sulphate was filtered through a Whatman 54 filter paper. The precipitate at 60% saturation was dissolved in 0.05M-Tris/HCl pH 7.4, containing 1.0M-NaCl, 0.01M-$CaCl_2$, 0.05% (v/v) Brij 35, and 0.03% (v/v) toluene. The preparation was then dialyzed against this buffer (100 volumes) for 2 days with 2 changes. The solution was clarified by centrifugation at 40,000 x g for 1 hour.

Concentration of various fractions containing proteolytic activity was performed in the early stages by ultrafiltration using collodion bags (Schleicher and Schuell, N.H., USA) with a nominal molecular weight cutoff of 10,000. At a later stage, ultrafiltration with the Immersible CX-10™ system (Millipore) with a 10,000 molecular weight cutoff, was used.

Activation of Conditioned Medium

In the early stages of this investigation the concentrated conditioned medium was treated with TPCK-trypsin (100µg/ml to 1mg/ml in assay buffer) and added to the conditioned medium at a concentration of 1µg/100µl of medium. The solution was left at room temperature for 10-15 minutes, and then a 5-10 fold molar excess of soybean trypsin inhibitor (1mg/ml in assay buffer) was added.

Trypsin bound covalently to agarose beads was also used to activate the proteinases. An aliquot of the trypsin-agarose was washed 3 times with assay buffer in a micro-centrifuge tube, and approximately 3 units of trypsin added per ml of proteinase solution. This was left at room temperature for up to 2 hours, with occasional hand inversion to resuspend the trypsin-agarose. The mixture was centrifuged, and the supernatant removed.

A third method of activation was used in association with the protocol of Cawston and Tyler (1979) as described above, but following the technique of Stricklin et al. (1983). Para-hydroxymercuribenzoate (pHMB), which was dissolved in 0.1N-NaOH at a concentration of 0.02M was added to the concentrated medium to give a final concentration of 0.001M. The mixture was incubated at 35°C for 2 hours, and stored at 4°C.

Gel Filtration of Concentrated Medium

At a later stage in this investigation, gel filtration at 4°C was routinely performed. For large scale preparations, aliquots (5-15 ml) of concentrated (25-50 fold) conditioned medium were applied to a column (2.5 x 115cm) packed with Bio-Gel agarose A0.5 and eluted with 0.05M-Tris/HCl pH 7.4 containing 0.05% (v/v) Brij 35, 0.03% (v/v) toluene with either 0.005M-$CaCl_2$ and 0.2M-NaCl or 0.01M-$CaCl_2$ and 1.0M-NaCl. For the chromatography of small volumes of proteinase solutions (1-2ml) a 1 x 115cm column was used. Both columns were calibrated for molecular weight estimation by the chromatography of protein standards and with ^3H_2O to determine total column volume.

C. Isolation and Purification of Collagen Substrates

Preparation of Soluble and Insoluble Bovine Skin Collagen

Acid soluble collagen (Type I) was prepared from the corium layer of a freshly flayed foetal bovine skin (Canada Packers, Edmonton) according to the protocol of Volpin and Veis (1971). All the extractant solutions contained the following in final concentration: 0.003M-sodium azide, 0.005M-NEM, 0.005M-benzamidine hydrochloride, 0.001M-6-amino hexanoic acid and 0.01M-EDTA. During the entire procedure, the skin or extractant solutions that contained the collagen were kept at 4°C.

The foetal bovine skin was washed in 10% (w/v) NaCl containing the inhibitors and left for 2 days in the above solution with one change. The hair and outer layers of skin, as well as any subdermal adipose tissue were scraped off, and then the skin was chopped into pieces of 2-3mm^3. These sections were suspended in the 10%-NaCl solution for several days with changes, and then suspended in 25%-NaCl for 24 hours. The salt was removed by extraction in water over several days.

Acid soluble collagen (Type I; Type III collagen is not extractable in dilute acids) was obtained from three 24 hour extractions with 0.5N-acetic acid. The extracts from each day were clarified by centrifugation (8,000 x g; 1 hour). The collagen in these extracts was precipitated by the addition of solid NaCl to a final concentration of 10% (w/v). After 24 hours, the precipitate was collected by centrifugation (17,000 x g; 1 hour). Additional NaCl (5%; w/v) was added to the supernatants to ensure maximum recovery of the collagen. The precipitated collagen was redissolved in 0.5N-acetic acid overnight, the solution clarified by centrifugation (17,000 x g), and collagen was reprecipitated by addition of an equal volume of 30% (w/v) NaCl. The precipitated collagen was recovered by centrifugation. The two steps were performed a total of 3 times, ending with the collagen in solution. The extracts from each day were handled separately to quantitate recoveries. The final collagen solutions were dialyzed

exhaustively against water without the proteinase inhibitors, lyophilized and stored at 4°C. This was called 'acetic acid soluble collagen'.

The residue after the three days of extractions was washed exhaustively with water over several days, lyophilized and stored at 4°C. This was called 'acetic acid insoluble collagen'.

The acetic acid soluble collagen was purified further for certain critical experiments to remove any bound proteinases (Pardo et al., 1980) by the method described by Gross (1958). The lyophilized collagen (10-40mg) was suspended in 0.5N-acetic acid at a concentration of 2mg/ml, and dissolved overnight at 4°C. The collagen solution was dialyzed against 2 changes of 0.1M-sodium phosphate buffer pH 7.6, and the concentration adjusted to approximately 1.0mg/ml with fresh phosphate buffer. Trichloroacetic acid (TCA; 25% solution in 0.45M-NaCl) was added to a final concentration of 2.5% and to pH of approximately 3.5 (never lower than pH 3). After 45 minutes the resultant precipitate was sedimented at 20,000 x g for 45 minutes at 4°C. The supernatant was dialyzed against 0.1M-sodium phosphate pH 7.6, for 48 hours with 2 changes. Ethanol (95%) was added to give a final concentration of 14% (v/v). The collagen was allowed to precipitate out of solution overnight and then sedimented at 30,000 x g. The collagen was resuspended in

0.5N-acetic acid, dissolved overnight, and then dialyzed against 0.01N-acetic acid. An aliquot was used for the measurement of the collagen concentration by the microbiuret assay as described below. Recoveries of the purified collagen were generally between 50-75%.

Preparation of Acetic Acid Soluble Rat Tail Tendon Collagen

Acid soluble collagen was also extracted from adult rat tail tendons. During the entire procedure the tissue and the extractant solutions that contained the collagen were kept cold. The tendons were removed, scraped, and then washed in 10% (w/v) NaCl containing 0.02%-sodium azide for 24 hours. The tissue was washed with water, and then defatted in chloroform/methanol (2:1), for 3 hours, followed by a wash with methanol and exhaustive washing with water. The tendons were suspended with stirring overnight in 0.5N-acetic acid containing 0.02% (w/v) sodium azide, and the undissolved residue removed by centrifugation (20,000 x g). Sodium chloride (30% w/v) was added to the supernatant to give a final concentration of 5%-NaCl, and this was left overnight. The precipitate was isolated by centrifugation (30,000 x g) and redissolved in 0.1N-acetic acid overnight. This solution was dialyzed exhaustively against 0.01N-acetic acid, and then against 0.5M-sodium phosphate buffer pH 7.1 for 2 days. The precipitate in the dialysis bag was recovered after centrifugation (30,000 x g), and redissolved in 0.1N-acetic acid, and then dialyzed against 0.01N-acetic acid. The

solution was clarified again by centrifugation (20,000 x g) and the supernatant lyophilized. An aliquot of this collagen was used for analysis of reducible cross-links after treatment with ^{3}H-sodium borohydride as described below.

Reduction of Acetic Acid Soluble Collagen

The acetic acid soluble fetal bovine skin collagen, that had been extracted with the proteinase inhibitors was further purified with TCA and ethanol precipitation as described above. This collagen, as well as the rat tail tendon collagen (not further purified) were kept on ice throughout the following procedure. The collagen, at a concentration of 1mg/ml in 0.01N-acetic acid, was neutralized by addition of 0.5M-sodium phosphate, pH 7.0, to a final concentration of 0.05M-sodium phosphate. Tritiated sodium borohydride was diluted with cold sodium borohydride initially in a 1:3 ratio, and in later experiments in a 1:1 ratio, and approximately 1mg of this was added to the collagen with mixing for 1 or 2 minutes. Different aliquots (5,10 or 20mg) of collagen were reduced in separate experiments with essentially the same result. Glacial acetic acid was then added to lower the pH to 5 and destroy the reductant. The solution was left for 30 minutes to allow for the tritiated gas to dissipitate, and then it was transferred to dialysis tubing and dialyzed against large volumes (4 litres) of 0.01N-acetic acid for several days with daily changes. In later experiments the collagen was

further dialyzed against 0.02M-sodium borate, pH 9.0 for 3 days, which significantly decreased the level of non-specific label on the collagen and then against 0.01N-acetic acid for 3 days. The collagen (either 0.5mg/ml or 1.0mg/ml) was dispensed in 1ml aliquots in 7ml scintillation vials and stored at -20°C.

^{14}C-Acetylation of Acetic Acid Soluble Collagen

An aliquot (10mg) of the acid soluble collagen extracted with proteinase inhibitors as described above, was dissolved in 0.1N-acetic acid at 4°C. The solution was dialyzed against 0.1M-sodium phosphate pH 8.8 for 2 days. The collagen (1.6mg/ml) was transferred to a thick walled container, and kept in a cold water bath to maintain the temperatures between 8 and 12°C throughout the following procedure. The contents of an ampule containing 500μCi of ^{14}C-acetic anhydride (119mCi/mMole), were diluted with 150μl of a 1% solution of cold carrier acetic anhydride in benzene, and transferred by washing with 350μl benzene. An aliquot of 60μl of this solution was added to the collagen at zero time and at every 30 minutes for a total of 5 additions. The solution was kept on ice for a further 1.5 hours. The acetylated collagen was then dialyzed against 0.01N-acetic acid for several days until no further radioactivity was released. The ^{14}C-collagen was stored at -20°C.

D. Assays

Glucose Utilization

The concentration of glucose in the conditioned medium was measured in order to determine the level of metabolic activity of the gingival explants in culture. A test kit for glucose determination (Auto/Stat Kit; Pierce Chemical Co.) was used. The procedure involved mixing reagents to obtain a 'working color reagent' containing 4-amino antipyrine, 2-4 dichlorophenol, glucose oxidase and horseradish peroxidase. The 'working color reagent' (4.0ml) was added to the samples (10μl), to be tested. The tubes were heated to 37°C for 15 minutes, cooled to room temperature, and optical density at 515nm measured within 10 minutes. The glucose concentration was read from a standard curve, with the use of the glucose supplied with the kit.

Lactate Dehydrogenase

The concentration of lactate dehydrogenase in the conditioned medium is related to the extent of cell death. This assay was performed using a lactate dehydrogenase kit (Sigma Kit #226-UV). The reaction, which was monitored at 340nm, was as follows:

Lactate + NAD ↔ Pyruvate + NADH

The rate of production of NADH is proportional to the LDH

concentration. The method involved the addition of 31ml of LD-L Reagent B (contains L-lactate) to LD-L Reagent A (β-NAD; lyophilized) with stirring. An aliquot (3.0ml) of this prepared reagent was pipetted into a 1cm cuvette, to which 100μl of sample was then added with mixing. The absorbances at 340nm at zero time and at 3 minutes were recorded with water as the reference. The entire reaction was performed at room temperature (approximately 20°C). The calculations were as follows:

A/3 = Final A - Initial A

LD-L(Unit/litre) = A/3 x 1660 x TCF

TCF (Temperature Correction Factor) at room temperature is 2.25. 'A' is the absorbance at 340nm.

Hydroxyproline

Hydroxyproline was estimated by the method of Bergman and Loxley (1970). Samples, to be analyzed were hydrolyzed in 6.0N-HCl, for 18-22 hours, dried and reconstituted in 500μl of water. Aliquots, in duplicate, were made up to 500μl with water, and to each, 1.0ml of isopropanol was added. This was followed by the addition of 0.5ml of the oxidant solution (see below) with vigorous vortexing. After 4 minutes ±15 seconds, at room temperature, 1.0ml of Ehrlich's reagent (see below) was added with mixing, and then the samples were heated to 60°C for 21 minutes in a

water bath. After one hour at room temperature to allow the color to fully develop, the optical density at 562nm was measured. Hydroxy-L-proline in aqueous solution was used as a standard, and stored at -20°C.

Oxidant solution: 4 volumes of a solution containing: 0.42M-sodium acetate anhydrous, 0.13M-trisodium citrate dihydrate, 0.026M-citric acid monohydrate, and 385ml of isopropanol made up to 1 litre with water; mixed with 1 volume of a 7% (w/v) solution of chloramine T in water. The solution was prepared just before each set of determinations.

Ehrlich's reagent: 17.6g of p-dimethylaminobenzaldehyde in 40.8g of 60% perchloric acid (specific gravity 1.54), and made up to 100ml with isopropanol was also prepared fresh.

Microbiuret Assay

The micobiuret method as described by Itzhaki and Gill (1964) was used for the estimation of collagen concentration. To an aliquot (50-500µl) of sample, made up to 500µl with water, 250µl of the biuret reagent (30% (w/v) NaOH, 0.21% (w/v) copper sulphate ($CuSO_4.5H_2O$) was added with mixing. After 30 minutes at room temperature the optical density at 300nm was measured. Gelatin (0.5mg/ml), of known moisture content, which was heated to 60°C for 30 minutes and cooled to room temperature, was used to obtain a

standard curve.

Collagenase Assay

The semiquantitative method of Berman et al. (1973) was used. Non-heparinized glass capillary tubes (1 x 75mm, Dade Capilets™) were partially filled, by capillary action, with a degassed solution of fetal bovine skin acetic acid soluble collagen (1mg/ml) adjusted to pH 7.5 and containing in final concentration 0.05M-Tris/HCl, 0.2M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide. Tubes were sealed at one end with Seal Ease™ (Clay Adams, USA) and at the other end with a removable short length of plastic tubing plugged with dental wax. The collagen was then left overnight at 35°C to gel. Samples to be tested for collagenase activity, were first adjusted to the same molarity of salt (0.2M-NaCl) as that of the collagen gel, and then applied (5µl), with a Hamilton 10µl syringe, to the tops of the now opaque collagen gels. The tubes were sealed and the rate of clearing (mm/hr) was measured at various intervals; if necessary for as long as 1 to 2 weeks.

In the latter part of this investigation a collagenase assay was used which was based on the conversion of α_1 to $\alpha_1$3/4 peptides, measured after electrophoresis of the products of digestion of acid soluble collagen with conditioned medium (modified from the protocol described by Sodek et al., 1981). The areas of these peptide peaks were measured, the $\alpha_1$3/4 peptide value adjusted by a factor of

4/3 to account for loss of the $\alpha_1$1/4, and the areas that represented α_1 chains, summed. The ratio, expressed as a percent of $\alpha_1$3/4 (adjusted) to the total area was related to the amount of collagen that was initially present in the incubation, and units of activity based on the amount of collagen degraded per hour were calculated (see Results).

Non-Specific Neutral Proteinase Assay #1

Gelatinase-like activity was estimated as described by Pettigrew (1978). In 1.5ml micro-centrifuge tubes, approximately 2.5mg of insoluble Azocoll was mixed with an aliquot of sample (25-500µl), and made up to 1.5ml with buffer to a final concentration of 0.05M-Tris-HCl, pH 7.5, 0.2M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide. The samples were incubated at 35°C, for 20 hours, or longer if necessary, on an end-over-end shaker set at the slowest speed. The mixtures were then centrifuged at 12,000 x g, for 3 minutes, the supernatant removed and the optical density at 520nm measured in a 1cm pathlength cuvette. A standard curve was constructed with TPCK-treated trypsin (0.5-5.0ng).

Non-Specific Neutral Proteinase Assay #2

The ability to digest azocasein was determined essentially as described by Sapolsky et al. (1974). Azocasein was dispersed in assay buffer at a concentration of between 5-10mg/ml, and heated to 56°C, for 30 minutes to solubilize it. Aliquots containing 2mg of azocasein, were

dispensed into 1.5ml micro-centrifuge tubes, and enzyme solution was added. The volumes were made up to 1.0ml with buffer (final concentration 0.05M-Tris/HCl, pH 7.5, 0.2M-NaCl, 0.005M-$CaCl_2$ and 0.02% (w/v) sodium azide). The reaction was carried out at 35°C for 20-24 hours. Trichloracetic acid (25% w/v) was added to a final concentration of 5%, and the precipitate of undigested azocasein sedimented at 12,000 x g. The supernatants were withdrawn quantitatively, and dispensed into 1.5ml micro-centrifuge tubes. Sodium hydroxide to a final concentration of 0.5M, (which increases the pH of the solution and enhances the color) was then added with shaking. The solution was again clarified by centrifugation. The optical density at 450nm was then measured. A standard curve was constructed with TPCK-treated trypsin.

Determination of Telopeptidase Activity by Gel Permeation Chromatography

The assay to determine the telopeptidase activity, (the activity that releases the extra-helical regions of collagen) underwent several modifications during the investigation. The procedure that was finally adopted was as follows:

In 1.5ml micro-centrifuge tubes, an aliquot (10 or 20µl) of ^{3}H-collagen (NaB^3H_4 reduced acid soluble fetal bovine skin collagen, purified by TCA and ethanol precipitations) was mixed with an equal volume of double strength assay buffer

(0.05M Tris/HCl, pH 7.5, containing 0.2M-NaCl, 0.005mM-$CaCl_2$ and 0.02% sodium azide). An aliquot (10-200µl) of the sample to be tested was added, and the solution made up to 240µl final volume with assay buffer, or with buffer that would normalize the concentration to that of the assay buffer constituents. The solutions were incubated at 18-23°C for a period that was dependent upon the estimated level of activity. This varied from 5 to 48 hours, but the majority of the incubations were for 20 hours. Concentrated formic acid (17µl) and 50µl of bovine serum albumin (4mg/ml in 1.0N-formic acid), were added and the solution chromatographed on a column (0.7 x 4cm; 2ml total volume; Bio-Rad Laboratories), packed with Bio-Gel P10 (nominal fractionation range 1,500-20,000), which was washed initially with 15ml of water and 5ml of 1.0N-formic acid. The columns were eluted with 1.0N-formic acid and the effluent collected in 20ml scintillation vials and counted after addition of 10ml of scintillation fluid, as described below. The five fractions that were collected were representative of the three regions of interest:

Fraction#	Volume(ml)	
1	1.3	Vo fraction;
2	0.2	
3	0.2	
4	1.0	Vt fraction;

5 1.0 Vt fraction;

The levels of radioactivity in fractions 2 and 3 were indicative of the success of the separation of the large and small molecular weight peptides.

Large Scale Incubations with Acid Soluble Collagen

Different methods were followed for the incubations of conditioned medium or of the enzyme preparations with non-radioactive acetic acid soluble collagen. Essentially, aliquots of collagen (80-160µg/ml final concentration, unless otherwise indicated) were incubated with the proteinase solutions in assay buffer (see above and Results) for varying periods of time at temperatures between 18-25°C. After addition of EDTA (final concentration of 0.025-0.05M) the incubation solutions were dialyzed against water at 4°C and lyophilized. The products were analyzed directly by electrophoresis on 5% polyacrylamide SDS gels, or digested with cyanogen bromide (see below) and examined by electrophoresis on 12% polyacrylamide SDS gels (below).

Incubations with Acetic Acid Insoluble Collagen

Aliquots (25-30mg) of lyophilized acetic acid insoluble collagen were rehydrated in 0.1N-acetic acid, and washed at 4°C, for 24 hour periods with 0.1N-acetic acid (twice), water (once), and twice with assay buffer (see above). All solutions contained 0.02% (w/v) sodium azide. The pieces of

insoluble collagen were sliced into 40μm sections on a freezing microtome, and the sections transferred into 1.5ml micro-centrifuge tubes. Aliquots of the proteinase solutions were added and the volume made up to 1.2ml with assay buffer. The mixtures were incubated at 25°C, for 4 days in a shaking water bath, after which EDTA, to a final concentration of 0.025-0.05M, was added. In later studies incubations were performed for 2 days at 22°C. The mixtures were centrifuged at 20,000 x g, for 60 minutes at 4°C, and the supernatants withdrawn (labeled S-1). The collagen residues were resuspended in 1.0ml water, and after 1 hour, the mixtures were recentrifuged and supernatants withdrawn (labeled S-2). The collagen was resuspended in 0.1N-acetic acid, and left overnight at 4°C, either stirring or on an end-over-end shaker. This supernatant, after centrifugation (30,000 x g for 90 minutes), was labelled S-3. Aliquots of the above 3 supernatants, were hydrolyzed with HCl (final concentration of 6N) and duplicate samples were analyzed for hydroxyproline. The solutions were dialyzed against water, or in later studies, against 0.02N-acetic acid and water, and lyophilized. Samples were electrophoresed on 5% polyacrylamide SDS gels or digested with cyanogen bromide and electrophoresed on 12% polyacrylamide SDS gels as described below.

To obtain more information on the nature of the solubilized collagen, the lyophilized samples were reconstituted in 0.05M-Tris/HCl, pH 7.5 containing

1.0M-$CaCl_2$ and chromatographed on a column (1 x 90cm) packed with agarose A1.5M (Scott and Veis, 1976a). Fractions that corresponded to the α1/4 peptides (see below) were pooled and desalted on columns (1.5 x 6cm) packed with Bio-Gel P4, that were previously equilibrated in the running buffer (1.0N-formic acid). These samples were lyophilized and digested with cyanogen bromide as described below. Aliquots of these cyanogen bromide digested α1/4 peptides were electrophoresed on 12% polyacrylamide SDS gels (see below). Well-characterized cyanogen bromide digests of acid soluble collagen were used as standards for comparisons of relative mobilities.

E. Polyacrylamide Gel Electrophoresis

SDS Polyacrylamide Gel Electrophoresis (5% and 7.5%)

Polyacrylamide gel electrophoresis was performed as described by Furthmayr and Timpl (1971), as modified by Scott and Veis (1976a). Stock solutions of the following were prepared, filtered and stored. A concentrated stock buffer consisted of a 0.4M-sodium phosphate, 0.3M-sodium hydroxide, 0.8% (w/v) sodium dodecyl sulphate (SDS) adjusted to pH 7.2 with 2N-NaOH, and stored at room temperature. The electrophoresis running buffer was a 1:8 dilution of the stock buffer. The stock acrylamide solution consisted of 29.1% (w/v) acrylamide and 0.9% (w/v) BIS stored in the dark at 4°C. The sample buffer was 0.01M-sodium phosphate

containing 0.22% (w/v) SDS and 2.0M-urea and stored at 4°C. The tracker dye, 0.05% (w/v) bromophenol blue in water, was stored at 4°C.

For the preparation of 5% polyacrylamide gels, 5ml of the acrylamide monomer solution was mixed with 3.75 ml of stock buffer and the volume made up to 28.4ml with water. Ammonium persulphate (1.5ml of a 5mg/ml solution freshly prepared), and TEMED (45µl) were added with mixing. The solution was immediately dispensed into glass tubes (0.55 x 12.5cm) filled to 10.5cm. N-butanol was applied to the top of each gel, and the gels left for 45 minutes to polymerize. Preparation of 7.5% polyacrylamide gels was as above except that 7.5ml of acrylamide was used.

Samples to be analyzed were weighed (20-30µg/gel) and dissolved in 40µl of sample buffer, and heated to 50°C for 30 minutes. Bromophenol blue (10-15µl) was added to each sample, and 20-25µl of this solution (in duplicate) was applied on top of each polyacrylamide gel under the running buffer.

Electrophoresis was carried out for 45 minutes at 1mA per tube, or until the samples had entered the gels, then at 3mA/tube for 15 minutes and finally at 6mA/tube for approximately 3.5 hours or until the tracking dye was within 1cm of the end of the gel. The gels were then carefully reamed out with water using a syringe fitted with a 4cm 26 gauge needle and India ink was injected into the center of the tracker dye band. The gels were stained

overnight in a solution of 25% (v/v) isopropanol, 10% (v/v) acetic acid, with 450mg Coomassie Blue, made up to 1 litre with water. The gels were destained for 4 hours in a solution containing 10% (v/v) isopropanol, 10% (v/v) acetic acid and 35mg of Coomassie Blue, and then for a further 24-36 hours in a diffusion destainer containing the ion-exchange resin AG501-X8(D), 20-50 mesh, and 10% (v/v) acetic acid. At a later stage in the investigation the intermediate destaining step was eliminated and on occasion, total time for staining was reduced to 5 hours (for the 5% polyacrylamide gels only).

SDS Polyacrylamide Gel Electrophoresis (12%)

This system employs the use of a stacking gel (3% or 5%) on top of a 12% separating gel, according to the method of Laemmli (1970). Stock solutions of the following were prepared, filtered and stored. The separation gel buffer (4 x concentrated) consisted of 1.5M-Tris and 0.4% (w/v) SDS and was adjusted to pH 8.8 with HCl. The stacking gel buffer (4 x concentrated) contained 0.5M-Tris, 0.4% (w/v) SDS and was adjusted to pH 6.8 with HCl. The running buffer (8 x concentrated) consisted of 0.2M-Tris, 1.536M-glycine, 0.8% (w/v) SDS and was adjusted to pH 8.3 with HCl. The sample buffer was 0.0625M-Tris/HCl, 2% (w/v) SDS, 2.0M-Urea, adjusted to pH 6. The catalyst, stock acrylamide, tracker dye and staining and destaining solutions were as for the 5% and 7.5% SDS-polyacrylamide gels.

Stock acrylamide (12ml) was mixed with 7.5ml of separation buffer, and the volume made to 29ml with water. After degassing, TEMED (30µl) and ammonium persulphate (1.0ml of a 5mg/ml solution) were added with mixing, and the solution immediately dispensed into gel tubes. Water or butanol was layered on top of the gels. After 30 minutes to allow for complete polymerization, approximately 0.2ml of stacking gel solution (1ml of acrylamide, 2.5ml of stacking gel buffer, 0.5ml of ammonium persulphate, and 15µl of TEMED and the volume made to 10ml with H_2O) was applied to the top of the separation gel.

Lyophilized samples were dissolved in the sample buffer with heating to 50°C for 30 minutes and bromophenol blue was (10-20µl) added to each solution. After layering samples under the running buffer, electrophoresis was performed with constant current initially (1mA/gel) until the sample had entered the gel, and then under constant voltage, at either 50 volts for 8 hours at room temperature, or 35-40 volts for 16 hours at 4°C. The gels were then processed as described above.

Scanning of Polyacrylamide Gels

The polyacrylamide gels after staining were exhaustively destained as described, and then scanned at 560nm in 10% acetic acid in an automatic Gilford Linear Transporter on a Beckman DU Gilford Spectrophotometer. The scans were recorded on a Fisher Scientific 'Recordall' chart

recorder. The relative mobilities of the stained peptides were calculated when necessary, by relating the distance travelled from the origin to the center of peak (peptide band) on the gel scan, to the distance travelled by the tracker dye. Quantitation of the relative areas of certain peptide bands was generally performed by multiplying the peak height by the width at half peak height. The apparent molecular weights (Mr) of the peptides were calculated from a standard plot of relative mobility versus the $\log_{10}$ of the molecular weight. To minimize any variation between runs, and between gels themselves, a standard plot was obtained on each gel of interest. For the cyanogen bromide peptides, α_1CB7 (24,900), and α_1CB3 (13,480) were used to estimate the molecular weight of peptides from the α_1 chain, and α_2CB3,5 (60,540) and α_2CB4 (29,500) were used for peptides derived from the α_2 chain.

Analysis of Polyacrylamide Gels for Radiolabelled Peptides

To detect radiolabelled peptides, polyacrylamide gels were frozen with solid CO_2, and sliced into 1mm sections with a Brinkmann Gel Slicer (Rexdale, Ont). These sections were placed individually into glass scintillation vials, to which 500µl of 0.6N-N.C.S.® Tissue Solubilizer (Amersham/Searle) was added. After incubation overnight at 35°C, 15µl of glacial acetic acid was added to each vial, followed by 10ml of Liquifluor® solution (42ml of Liquifluor (New England Nuclear) mixed with 1 litre of toluene). After

24 hours in the dark, to allow chemiluminescence to decay, the radioactivity was measured.

Pre-elution of Gels

When it was desired to isolate peptides separated by gel electrophoresis, the gels were initially pre-eluted. After preparation in the tubes, the gels were reamed carefully out of the tubes, washed over several days with water obtained from the Milli-Q system (see Materials), at 4°C, and then dialyzed against electrophoresis running buffer. The gels were reinserted into the glass electrophoresis tubes and the lower end of each was sealed with dialysis membrane. The electrophoretic conditions were as previously described.

Extraction of Peptides from the Gels

The method described by Scott (1982) was followed. The protein containing zones in the gels were excised, macerated by forcing through a 23 gauge syringe needle and eluted into 4-5 volumes of 25% (v/v) pyridine overnight at 37°C. Extraction was repeated and the extracts were pooled. The protein was separated from the dye (Coomassie Blue) by chromatography on a column (1.5 x 6cm) packed with Bio-Gel P10 (100-200 mesh). The samples were eluted with 1N-formic acid and lyophilized.

F. Other Techniques

Affinity Chromatography

Attempts were made to purify proteinases by collagen-affinity chromatography. The initial attempts involved neutralizing acid soluble collagen (1mg/ml) to pH 8.5 with a final concentration of 0.1M-sodium bicarbonate containing 0.2M-NaCl ('coupling buffer'; Bauer et al., 1971). The extent of binding was determined by the incorporation of ^{14}C-collagen (100µg). CNBr-activated Sepharose 4B (1-2g; Pharmacia), was initially washed with 300-600ml of ice cold 0.001N-HCl, (over a 15 minute period) on a sintered glass filter, washed briefly with the coupling buffer, mixed with the collagen solution in a glass culture tube, and incubated overnight at 4°C, on an end-over-end mixer. The gel was sedimented (200-300 x g; 20 seconds) and supernatant withdrawn. An equal volume of 1N-ethanolamine (pH 9) was added to block residual active groups. After 2 hours at 4°C, the collagen-Sepharose was alternately washed with 0.1M-sodium acetate buffer, pH 4, containing 0.5M-NaCl, and 0.1M-sodium bicarbonate, pH 8.5, containing 0.5M-NaCl. An aliquot of the collagen-Sepharose gel was removed and radioactivity measured.

In later experiments, due to problems with the collagen precipitating out of the solution, 0.05M-sodium citrate, pH 6.3, containing 0.2M-NaCl was used as the coupling buffer.

Conditioned medium was prepared by dialysis overnight against the standard assay buffer containing no sodium chloride, and applied to the collagen-Sepharose column (0.7 x 4 cm). The columns were eluted in various ways (see Results).

Amino Acid Analysis

A. Acid Hydrolysis

The samples to be analyzed were mixed with an equal volume of 12N-HCl (redistilled), or if dried initially, dissolved in constant boiling 5.7N-HCl (Pierce Chemical Co.). Phenol (5% w/v) in water was added to a final concentration of 0.4%. Tubes were evacuated and sealed, and then incubated at 105°C, for 18-22 hours. Solutions were dried and reconstituted with 0.001N-HCl, and an aliquot taken for amino acid analysis.

B. Base Hydrolysis

The samples to be analyzed were lyophilized, dissolved in 50µl of 2N-NaOH, and transfered to a specially designed teflon block. The samples were hydrolyzed as above, HCl was added to lower the pH to 2-3, and volume made up to 0.5ml with H_2O. An aliquot of this was taken for amino acid analysis.

Aliquots of the hydrolysates were analyzed on a Beckman 121MB analyzer, using the recommended program for collagen

hydrolysates (Beckman DS-526). The limit of sensitivity for hydroxyproline was approximately 0.25nmoles or lower. This amount of hydroxyproline was equivalent to approximately 240ng of collagen.

For the detection of ^{3}H-labelled amino acids, the effluent from the analyzer was collected directly into scintillation vials (2 minutes, 0.5ml per fraction). Water (0.5ml) and scintillation fluid (10ml) were added and the radioactivity measured as described below.

Liquid Scintillation Counting

Radioactivity was measured with a Philips Liquid Scintillation Counter (model PW4700). Aqueous samples were mixed with 4ml of ReadiSolv™ (Beckman), or Scintiverse™E (Fisher) in 7ml vials, or with 10ml of scintillation fluid in 20ml vials. When necessary, to avoid precipitation due to high salt concentrations, the samples were diluted with water.

Dialysis

Dialysis tubing (Spectrapor™, USA) with a 6,000-8,000 molecular weight cutoff was soaked and washed in distilled water, and then boiled in water for 15 minutes. The tubing was cooled, rinsed extensively with distilled water and used. All dialysis was performed at 4°C, and involved at least 2 changes of the dialysis solution, each being of 24 hour duration unless otherwise indicated.

Cyanogen Bromide Digestion

The samples to be digested, if in solution, were made up to 70% formic acid with the addition of an appropriate amount of concentrated formic acid. The solution was flushed with nitrogen for 2 minutes and cyanogen bromide to a final concentration of 12-20mg/ml was then added. If the sample was dry initially, cyanogen bromide in 70% formic acid was added, and the solution was briefly flushed with nitrogen. The digestion was carried out at 25-30°C for 4 hours with stirring. The digests were then diluted with a large excess of water and lyophilized. The samples were redissolved in water and lyophilized again to remove all traces of the cyanogen bromide and volatile reaction products. The products of digestion were analyzed by electrophoresis of the samples on 12% polyacrylamide gels (described above).

III. RESULTS

A. Porcine Gingival Explant Cultures

The conditioned medium from cultures of porcine gingival explants was examined for levels of collagen digesting (or collagenase) and Azocoll digesting activities as well as for the concentrations of lactate dehydrogenase and glucose (Fig 3). The effects of bacterial collagenase on the collagen substrate, and trypsin on the insoluble Azocoll are shown in Fig 4. Due to the presence of significant levels of tissue inhibitors as reported by Pettigrew et al. (1978), as well as the demonstrated high levels of lactate dehydrogenase activity in Day 1 culture medium (Fig 3d), this medium was discarded. The lactate dehydrogenase activity from the fourth day in culture and onwards was significantly increased over the relatively low levels of Days 2-3. The Azocoll-digesting and collagenase activities (non-artificially activated) present in conditioned medium from Days 6-10 were also assayed (results not shown). The activities decreased to approximately 50% of the levels determined for Days 2 and 3, by Day 10. In addition, the gingival explants themselves appeared to be undergoing rapid decomposition after the fifth day in culture. Thus conditioned medium from Days 2-4, or occasionally in the earlier studies, Days 2-5, were used in this study to minimize contamination with intracellular contents released upon cell death.

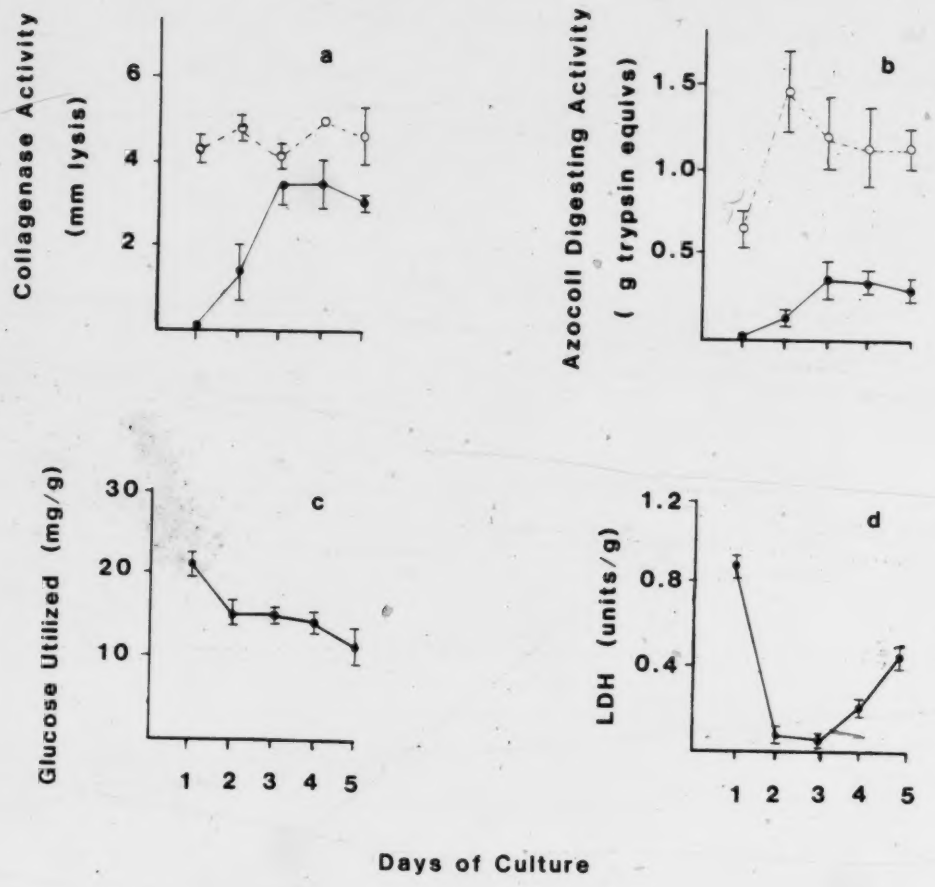


Figure 3. Analysis of Gingival Explant Culture Medium.

An aliquot of conditioned medium from each day of culture of porcine gingival explants was analyzed for:

a) clearance of collagen gels in capillary tubes; after incubation for 24 hours at 35°C, the level of digestion (clearance) was measured; (●) non-activated; (○) activated with trypsin-agarose. The means of duplicate determinations and ranges of 1 flask are shown.

b) digestion of Azocoll; incubation at 35°C for 24 hours; results expressed as trypsin equivalents per flask; (●) non-activated, (○) activated. The mean of duplicate determinations and ranges of 1 flask are shown.

c) level of glucose utilized per gram of tissue per 24 hours. Points shown are average values of 5 flasks (single determinations) with the Standard Error of Mean.

d) units of lactate dehydrogenase (LDH) per gram of tissue per 24 hours. Points shown are average values of 5 flasks (single determinations) with the Standard Error of Mean.

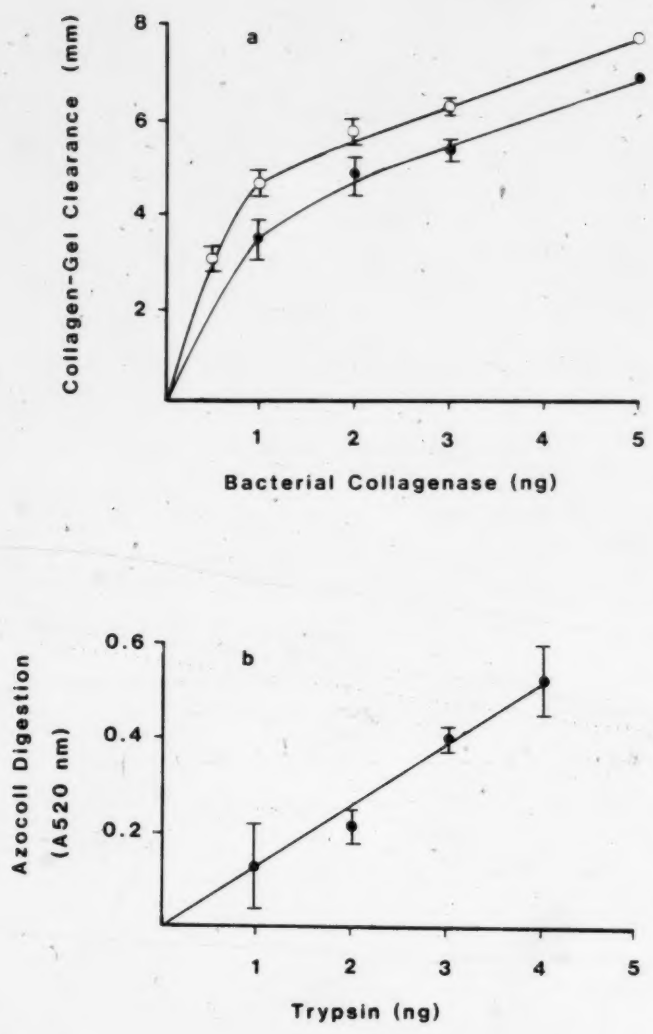


Figure 4. Digestion of Standard Substrates.

a) bacterial collagenase incubated at 35°C with reconstituted collagen in capillary tubes; level of clearance at 16 hours (●), and at 21 hours (○).
b) trypsin (TPCK-treated) incubated with Azocoll (2.0mg), in 1.0ml of assay buffer (0.05M-Tris/HCl pH 7.4, containing 0.2M-NaCl, 0.005M-$CaCl_2$, 0.02% sodium azide) for 24 hours at 35°C.

The mean of duplicate determinations and ranges are shown.

B. Incubation of Conditioned Medium with the Collagen Substrates

The presence of vertebrate collagenase was demonstrated by the electrophoresis, on 7.5% polyacrylamide gels, of the products of the incubation of conditioned medium with acetic acid soluble collagen (Fig 5). The presence of the bands labelled $\alpha 3/4$ and $\alpha 1/4$ were indicative of the action of collagenase. In addition, denatured acid soluble collagen was incubated at 37°C with the conditioned medium to demonstrate the presence of 'gelatinase' (Fig 5c). The digestion of this collagen gave rise to a heterogeneous population of collagen fragments.

Acetic Acid Insoluble Collagen Incubations

Concentrated conditioned medium, was incubated with acetic acid insoluble fetal bovine skin collagen to determine the level of solubilization. In a series of experiments, a significant amount of the insoluble collagen was solubilized even when the conditioned medium was not artificially activated (Table 1). Prior treatment of the conditioned medium with trypsin effectively doubled the amount of collagen solubilized. The addition of EDTA, even at a final concentration of 0.005M, effectively inhibited the release of collagen. A positive control for the release of collagen monomers was demonstrated by the incubation of elastase, isolated from polymorphonuclear leukocytes, with the substrate. In addition, concentrated conditioned medium

Figure 5. Gel Electrophoresis of the Products of Incubation of Acid Soluble Collagen and Conditioned Medium.

An aliquot (100µl) of concentrated (6 fold) conditioned medium (Days 2-4) was incubated with native and denatured collagen (450µg), in assay buffer (final volume 1.2ml), as described below. The products were electrophoresed on 7.5% polyacrylamide SDS gels.

a) no-enzyme control; incubated for 24 hours at 25°C.
b) native acid soluble collagen; incubated with the medium for 24 hours at 25°C.
c) denatured collagen (by heating to 60°C for 1 hour); incubated with the medium for 3 hours at 37°C.

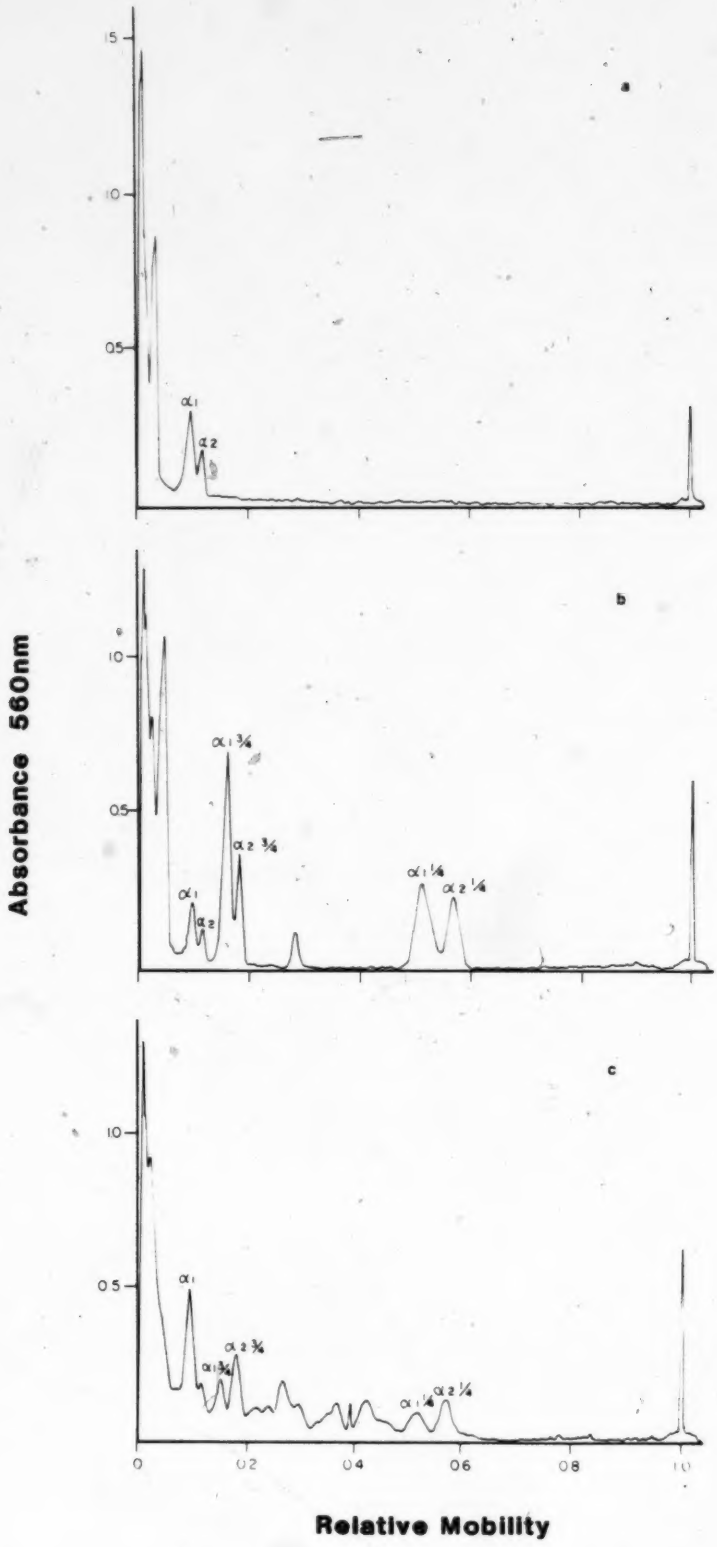

a
b
c
α1
α2
α1 ¾
α2 ¾
α1 ¼
α2 ¼
Absorbance 560nm
Relative Mobility
0
02
04
06
08
10
05
10
15

Table 1. Solubilization of Acid Insoluble Collagen.

Acetic acid insoluble collagen (26-28mg) was incubated with various preparations of conditioned medium (0.5ml) for 4 days at 22-25°C in assay buffer (final volume: 1.0-1.2ml). The solubilized collagen was quantitated by hydroxyproline analysis, and the results expressed as a percent of the starting dry weight of the insoluble collagen. The results below are grouped according to the different batches of conditioned medium tested against this substrate. The results are of single incubations unless ranges are shown and are thus in duplicate.

ENZYME PREPARATION	NEUTRAL SALT SOLUBLE(%)	ACETIC ACID SOLUBLE(%)	TOTAL (%)	CORRECTED TOTAL (%)
no-enzyme control	1.4	2.0	3.4	0
medium (6 x conc.)	6.5±0.6	5.5±0.7	12.0±1.3	8.6
medium (conc.,act.)	10.2	8.7	18.9	15.5
no-enzyme control	1.0	2.3	3.3	0
boiled enzyme control[a]	1.0	2.5	3.5	0
medium (6 x conc.)	3.6±0.3	2.5±0.3	6.1±0.6	2.8
PMN leuk. elastase	6.6	6.7	13.3	10.0
elastase and SBTI	2.5	4.4	6.9	3.6
no-enzyme control	1.8±0.1	2.0±0.1	3.8±0.2	0
EDTA (0.05M) control	1.8±0.1	2.5	4.3	0.5
medium (conc.,act.) & EDTA (0.005M)	2.7	1.6	4.3	0.5
medium (conc.,act.) & EDTA (0.05M)	2.6±0.1	1.9±0.3	4.5±0.4	0.7
medium (25 x conc., act.)	9.9±0.6	6.7±0.7	16.6±1.3	12.8

conc. - concentrated; act. - activated;
PMN leuk. - polymorphonuclear leukocyte;
SBTI - soybean trypsin inhibitor.

a - enzyme solution was heated to 95°C for 10 minutes.

was analyzed for hydroxyproline content. It was determined that the background levels due to non-diffusible collagen in the conditioned medium would represent not more than 0.1-0.2% of the total insoluble collagen in the incubations.

The products of digestion of insoluble collagen were examined by gel electrophoresis (Fig 6). The majority of the collagen that was extracted in the neutral salt and water washes was essentially in the form of $\beta 3/4$, $\alpha 3/4$ and $\alpha 1/4$ peptides (Fig 6b). In the acetic acid extracts only 40-50% of the collagen β and α chains appeared to be converted to the characteristic collagenase products (Fig 6c). However this difference in conversion, could be due to collagen which was inaccessible to the collagenase but which was extracted under acid conditions (collagen fibrils swell in acetic acid), as was shown for the no-enzyme control (Table 1; acetic acid extract). It was also observed from these electrophoretic profiles, that the relative amounts of the β and α components (β/α) did not appear to differ significantly from the no-enzyme controls. However the collagen that was solubilized by the action of the leukocyte elastase appeared to be made up primarily of α chains (Fig 6d), which is in agreement with the observations of Starkey et al. (1977).

According to the working hypothesis, outlined in the Introduction, another proteinase, or possibly, another proteolytic activity of collagenase could be necessary to initiate the degradation of fibrillar collagen. Such an

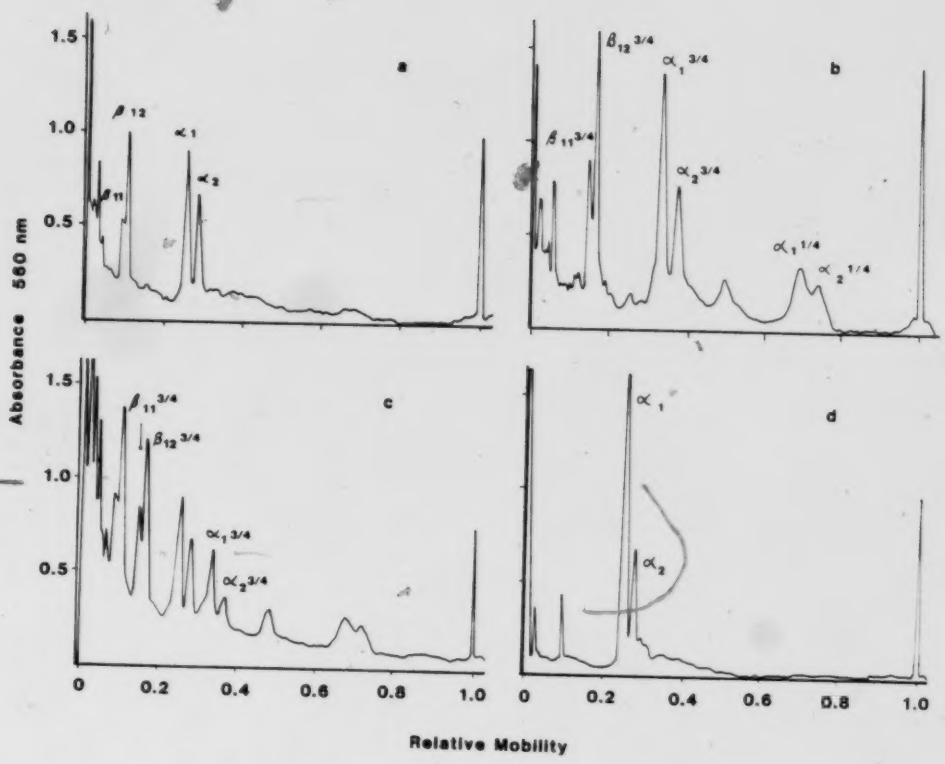


Figure 6. Gel Electrophoresis of the Products of Incubation of Acetic Acid Insoluble Collagen and Conditioned Medium.

Concentrated (6 fold) conditioned medium (0.5ml) was incubated with 27mg (+/- 2mg) of acetic acid insoluble collagen in assay buffer (final volume 1.2ml) for 96 hours at 25°C. The solubilized collagen was dialyzed against water, lyophilized, and electrophoresed on 5% polyacrylamide SDS gels.

a) no-enzyme control - neutral salt extract.
b) incubation with medium - neutral salt extract.
c) acetic acid extract of the collagen residue of (b).
d) incubation with polymorphonuclear leukocyte elastase (8µl) - neutral salt extract.

Note the almost complete absence of beta chains in the electrophoretic profile of the leukocyte elastase digested collagen.

activity could have its effect on fibrillar collagen by cleaving on the proximal side (i.e. between the cross-link site and the helix) of the intermolecular cross-links originating in the N and/or C-terminal extra-helical regions. However, according to the electrophoretic profiles (Fig 6b,c), it appeared that if there was an action at the N-terminus, it was not prominent. Nevertheless, activity against either of the telopeptide regions of the collagen molecules would likely cause release of collagen, either as monomers or as aggregates, which would then be subject for further breakdown by the action of collagenase. To determine if such cleavage had occurred, the loss of 10-25 amino acid residues from an α chain of 1050 residues would have to be detected.

Fractionation (by gel filtration) of the products of incubation of acid insoluble collagen and conditioned medium is shown in Fig 7. The fractions that contained the α1/4 peptides were pooled, desalted and digested with cyanogen bromide. The resultant digest was analyzed by electrophoresis on polyacrylamide SDS gels (Fig 8). A preparation of cyanogen bromide peptides from native acid soluble collagen, that was previously characterized in this laboratory, was used as a standard. The relative mobility of the major band (peak c) obtained from the test substrate was indistinguishable from that of the shortest form of α_1CB6 (α_1CB6c) from the cyanogen bromide digest of the control. This latter peptide, of an apparent Mr of 17,200, has been

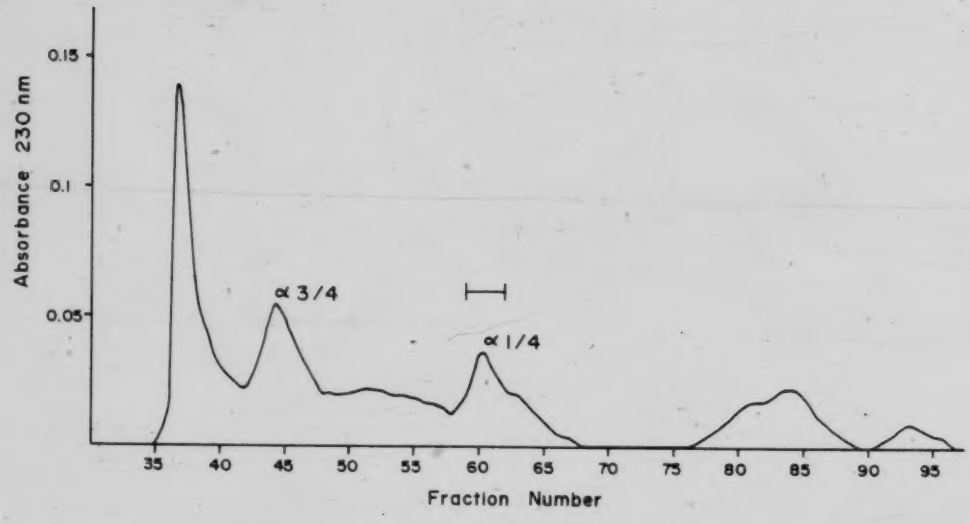


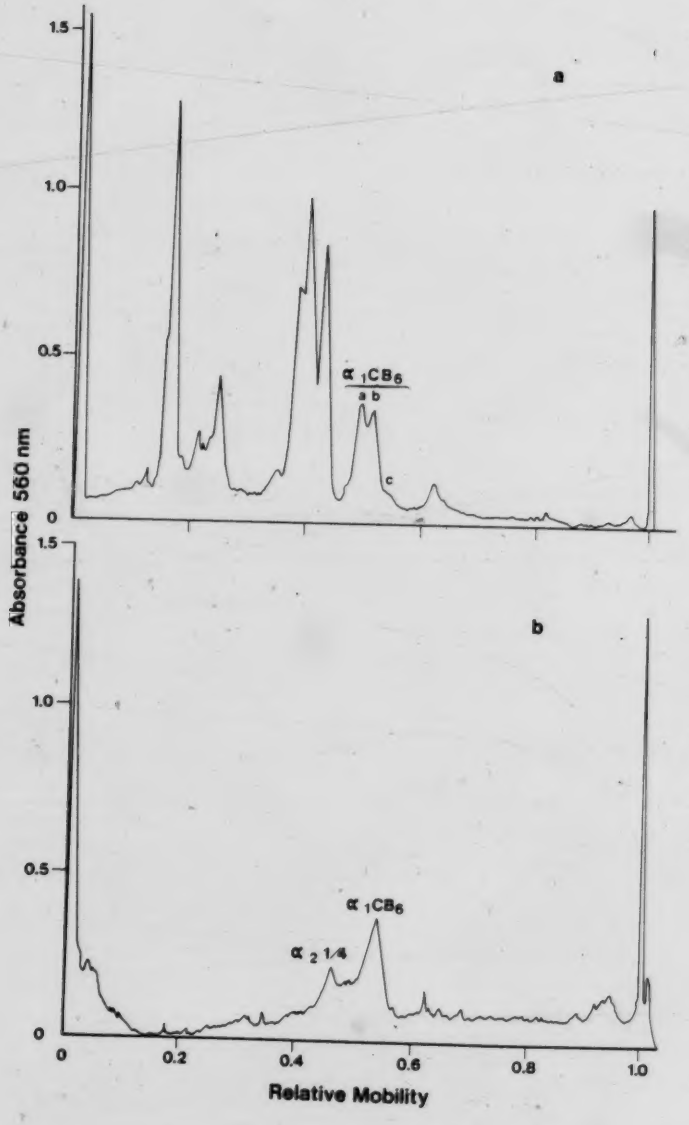
Figure 7. Elution Profile of the Collagen Released from the Incubations of Acid Insoluble Collagen and Conditioned Medium.

The collagen that was extracted in the neutral salt washes from the incubations of the insoluble collagen and conditioned medium (concentrated 6 fold, but not activated) was dialyzed and lyophilized. The sample was dissolved in 0.05M-Tris/HCl buffer pH 7.2 containing 1.0M-$CaCl_2$, and applied to a column (1 x 90cm) packed with agarose A1.5M, and chromatographed in the buffer described above. Those fractions (1.0ml each) that contained the $\alpha_1$1/4 peptides (59-62) were pooled, desalted and digested with cyanogen bromide as described in Figure 8.

Figure 8. Gel Electrophoresis of Cyanogen Bromide Digested α 1/4 Peptides.

The neutral salt extractable products of incubation of acid insoluble collagen and conditioned medium (Figure 6), were chromatographed on agarose A1.5M (Figure 7). Those fractions corresponding to α1/4 peptides were pooled, desalted in 70% (v/v) formic acid on columns (1.5 x 7cm) packed with Bio-Gel P4, and digested with cyanogen bromide. These products, as well as a cyanogen bromide digested control (acid soluble collagen known to contain intact C-terminal telopeptide), were electrophoresed on 12% polyacrylamide SDS gels.

a) control - acid soluble collagen; note the 3 forms of α_1CB6 (see text).
b) isolated α 1/4 peptides; note that the major peptide migrates with a relative mobility similar to that of α_1CB6c (control).


a
b
α 1CB6
a b
c
α 1CB6
α 2 1/4
Absorbance 560 nm
Relative Mobility
0
0.5
1.0
1.5
0.2
0.4
0.6
0.8

shown to be devoid of essentially the entire carboxy-terminal extra-helical region. The other α_1CB6 peptides that are seen in Fig 8a, contain either all of the C-terminal telopeptide region (α_1CB6a; apparent Mr of 19,600), or a portion of it (α_1CB6b; apparent Mr of 18,400).

A haemagglutination inhibition assay was performed on the collagen that was released during the incubations with acid insoluble collagen, using antibodies specific for the C-terminal determinant of the α_1 chain (residues C4 - C9; Table 2). In this assay the collagen (control) with intact C-terminal gives the highest decrease in titre. There appears to be a correlation between the level of solubilization and the relative proportion of this collagen with an intact C-terminal determinant, however any conclusions based on only this data would not be valid. Of the α_1CB6 peptides only the shortest form (α_1CB6c) would not have the determinants for these antibodies.

To confirm that the α_1CB6 peptide derived from the digested collagen was in fact lacking the C-terminal region, this peptide was extracted from the polyacrylamide gels as described in the Methods. Aliquots of the extracted peptide were then tested in a radio-immunoassay specific for the determinant within the carboxy-terminal extra-helical extension on the α_1 chain (by P.G. Scott). In an assay with a sensitivity limit of 20ng of peptide, an aliquot of 128ng of isolated α_1CB6 did not have detectable antigenic activity. Thus, three separate experimental approaches

Table 2. Haemagglutination Inhibition Assay of the Collagen Solubilized from Incubations of Acid Insoluble Collagen and Conditioned Medium.

Antibodies specific for the C-terminal determinant of the α_1 chain were used to demonstrate the correlation between the extent of solubilization and the relative amount of collagen lacking the C-terminal telopeptide region containing the determinant site. The results are grouped according to the experiments performed with two different batches of medium.

ENZYME PREPARATION	TOTAL SOLUBILIZED (%)[a]	TITRE DECREASE ($\log_2$)[b]
no-enzyme control	3.4	6
medium (6 x conc.,act.)	12.0	2
no-enzyme control	3.3	5
medium (6 x conc.)	6.1	4
acid soluble collagen	N.A.	7

conc. - concentrated; act. - activated;
N.A. - not applicable.
a - from Table 1.
b - single determinations

confirm that the collagen that was solubilized due to proteolytic activity of the conditioned medium was for the most part lacking the intact C-terminal telopeptide.

Acid Soluble Collagen Incubations

As described above, the collagen that was solubilized did not have an intact C-terminal telopeptide. However, to confirm that the proteolytic activity was in fact acting at the C-terminus of the cross-linked collagen was not possible using the fibrillar collagen substrate. Thus acid soluble collagen known to contain the C-terminal extra-helical region was used to demonstrate this activity.

The protocol that was followed in the initial attempts to demonstrate the cleavage of the C-terminal telopeptide was as described for the digestion of acid insoluble collagen. However there were a number of problems concerning the interpretation of the results, which were not understood at the time. Analysis of the products of incubations of acid soluble collagen and conditioned medium demonstrated on occasion protein material 5-15,000 daltons smaller than the α3/4 peptides (Fig 9b), as well as loss of resolution between the $\alpha_1$1/4 and $\alpha_2$1/4 peptides (not shown). These are likely due to extra cleavages of the collagenase-derived peptides, which, as determined at a later date, was probably caused by the use of excessive amounts of proteolytic activity, in combination with the temperature (25°C) and length (up to 4 days) of the incubations. The

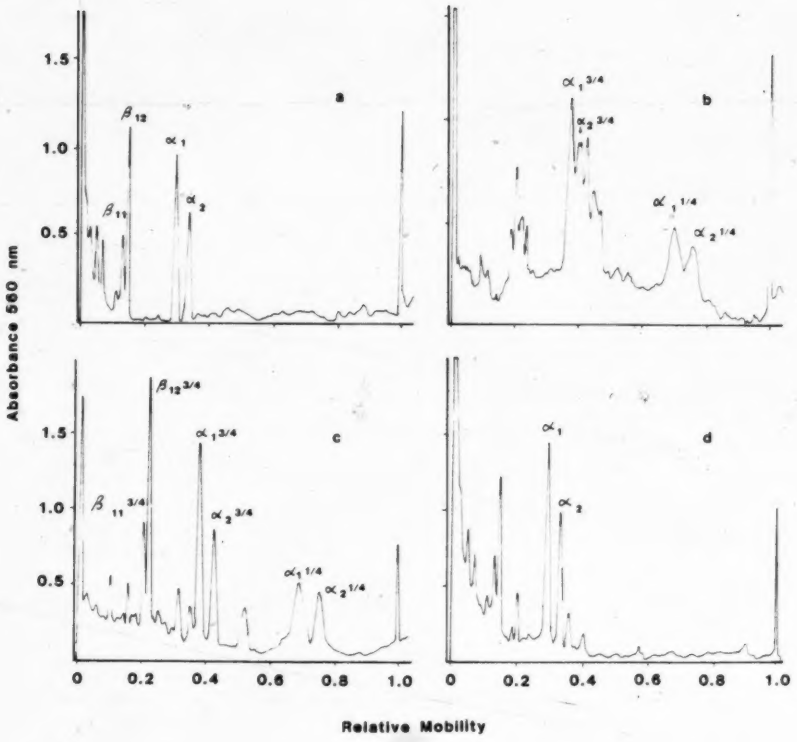


Figure 9. Gel Electrophoresis of Products of Incubation of Acid Soluble Collagen and Conditioned Medium.

Acid soluble collagen (750µg) was incubated in assay buffer (final volume 1.0ml) for 96 hours at 22-24°C with various preparations of conditioned medium, and analyzed by electrophoresis on 5% polyacrylamide SDS gels.

a) no-enzyme control.
b) concentrated (20 fold) conditioned medium (100µl) of untreated gingival explants (Day 3-4); Note the extra cleavages of the collagenase derived α 3/4 peptides.
c) concentrated (10 fold) conditioned medium (200µl) of cycloheximide-treated gingival explants (Day 3-4).
d) concentrated (10 fold) conditioned medium (200µl) of cycloheximide-treated gingival explants (Day 5); note the low level of conversion of α_1 to $\alpha_1$3/4.

'over-digested' collagen was not examined further.

It was demonstrated at the time that the conditioned medium obtained from Day 3-4 cultures treated with cycloheximide, which blocks protein synthesis, did not cause any extra cleavages when incubated with acid soluble collagen (Fig 9c). Under identical conditions, untreated gingival explant culture medium caused the anomalous digestion described above (Fig 9b). In addition, as shown in Fig 9d, medium from Day 5 cultures of cycloheximide-treated gingival explants had only traces of collagenase activity. Cycloheximide did bring about a significant reduction in the level of collagenase in the conditioned medium. As described in a later section, in the incubation of untreated conditioned medium with acid soluble collagen, there was probably a 100-200 fold excess of collagenase in terms of substrate to be digested. The collagen digest from the incubation with the conditioned medium (Day 3-4) of the treated explants was chromatographed on agarose A1.5M, and the protocol described for the acid insoluble collagen was followed. However, insufficient material was recovered at the final step for the analysis on gel electrophoresis. Various other attempts to improve recoveries of the $\alpha 1/4$ peptides were also without success.

C. Development of an Assay to Determine Proteolytic Activity Against the Terminal Extra-Helical Regions

A different approach was attempted to overcome the problems encountered in the interpretation of the results of digestions of acid soluble collagen. It is known that intermolecular cross-link formation in collagen is preceded by conversion of lysine and hydroxylysine (situated in the extra-helical regions) to lysinal or hydroxylysinal (Bornstein and Traub, 1979). These amino acid residues can be labelled by reduction of the aldehyde, and thus could act as markers for an enzyme activity which releases the region (or regions) containing the cross-linking site(s) of collagen. In ^{3}H-sodium borohydride-reduced soluble collagen (from tissues such as bovine skin, tendon) the main stable labelled compound would be ^{3}H-hydroxynorleucine (see Fig 10; Bornstein and Traub, 1979).

Characterization of the Radiolabelled Substrate

Aliquots of the ^{3}H-collagen were hydrolyzed with base or acid, and run on the amino acid analyzer (Fig 11). The ^{3}H-amino acid elution profile of the base hydrolysate show the presence of a major radioactive peak eluting near the position of glycine. According to a report by Mechanic (1974), this is probably ^{3}H-hydroxynorleucine which is derived from the ^{3}H-sodium borohydride reduction of the cross-link precursor - lysinal (Fig 10).

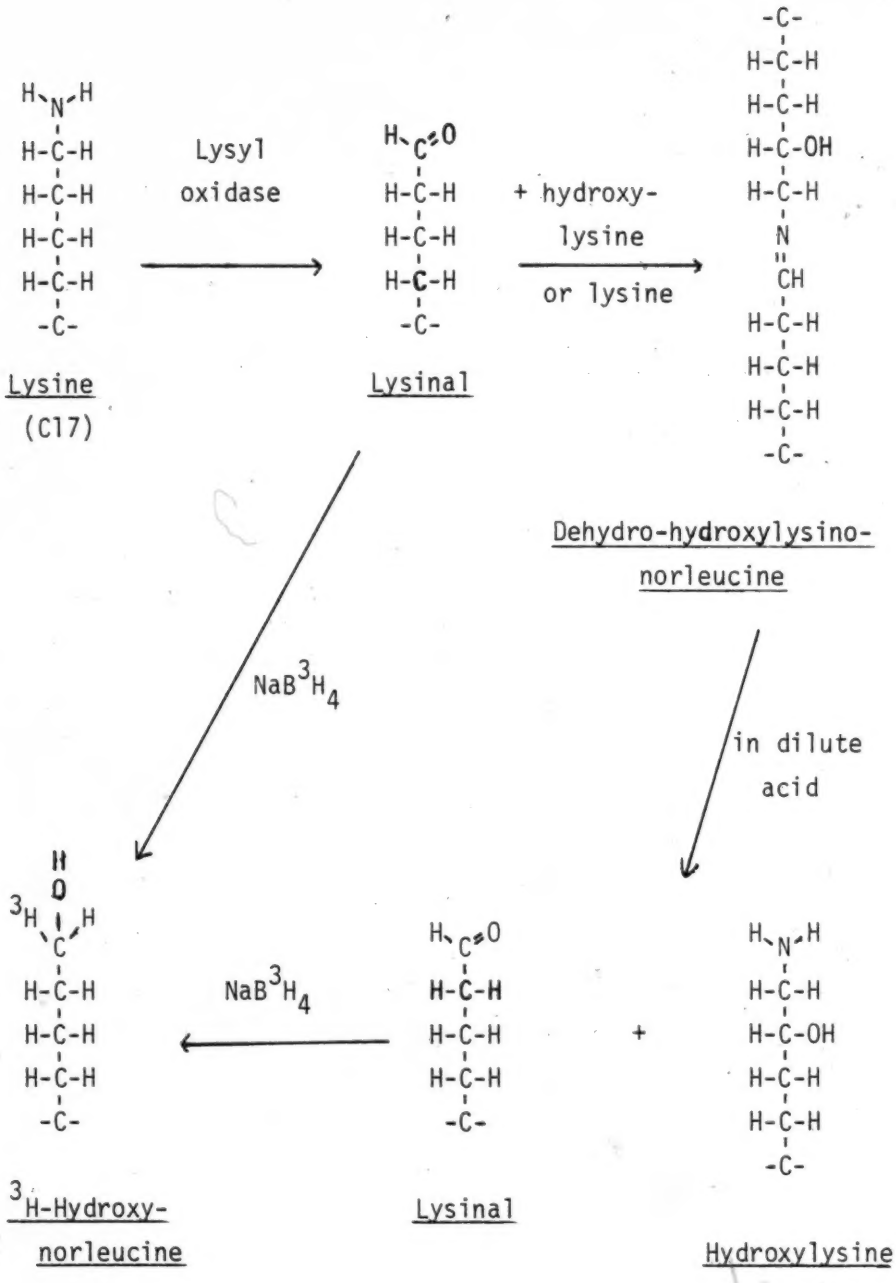


Figure 10. Reduction of Collagen Cross-link Precursors.

Acid soluble collagen which was further purified to remove non-collagenous proteins, was treated with ^{3}H-sodium borohydride. One of the products of reduction, ^{3}H-hydroxynorleucine is as shown above.

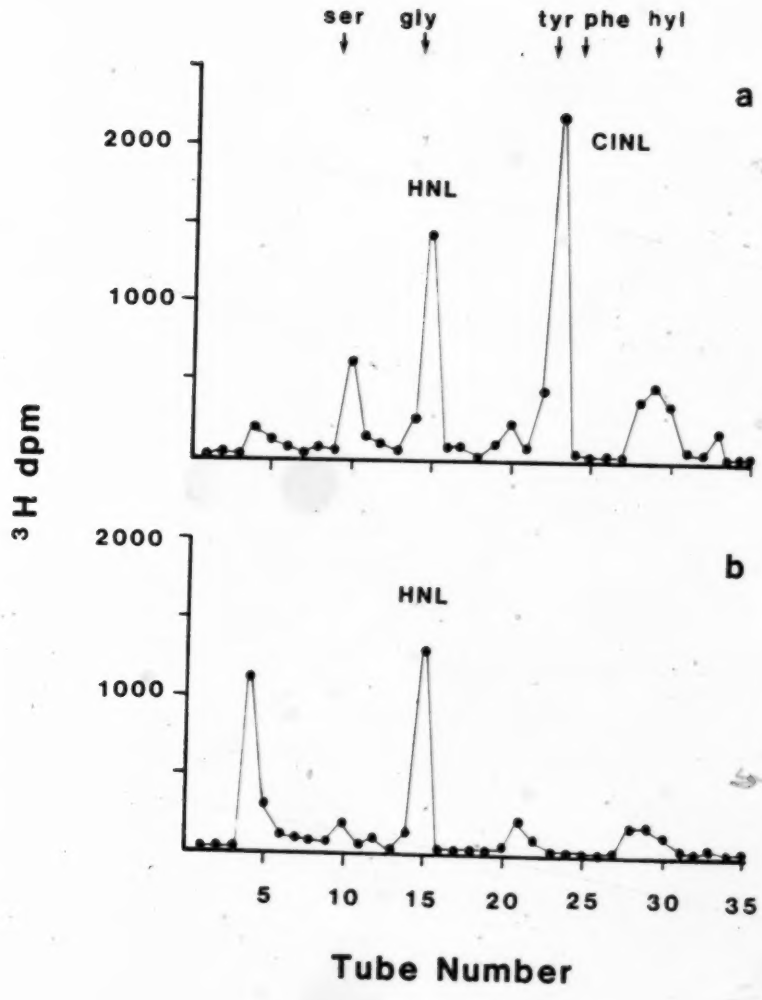


Figure 11. ^{3}H-Amino Acid Elution Profile of Sodium Borohydride Reduced Acid Soluble Collagen.

Tritiated collagen samples that were hydrolyzed in acid or base, were applied to the amino acid analyzer, fractions (0.5ml, 2 minutes) were collected in scintillation vials, and radioactivity measured.
a) acid hydrolyzed (5.7N-HCl) ^{3}H-collagen (21µg).
b) base hydrolyzed (2.0N-NaOH) ^{3}H-collagen (8µg).
(↓) elution positions of amino acids.
HNL - hydroxynorleucine; ClNL - chlornorleucine.
Fractions 4-5 contains non-retarded material.

The ^{3}H-amino acid elution profile of the acid hydrolysate was somewhat more complicated. There were two major peaks, one of which eluted near the position of glycine as shown for the alkaline hydrolysate, and a second peak eluting between tyrosine and phenylalanine. This latter labelled compound is likely to be a byproduct of hydrolysis in hydrochloric acid in which the chloride exchanges with the hydroxyl in hydroxynorleucine to form chlornorleucine (Bailey et al., 1970; Robins, 1976). In addition the sum of the radioactivity associated with ^{3}H-hydroxynorleucine and the putative ^{3}H-chlornorleucine peak in acid hydrolysates was equivalent to the amount of ^{3}H-hydroxynorleucine in base hydrolysates, when both were expressed as a function of phenylalanine concentration (Table 3).

A further check on the type of labelled amino acid co-eluting with glycine, involved the isolation and reduction of acetic acid soluble rat tail tendon collagen. This material is rich in the reducible cross-link precursor - lysinal (Deshmukh, 1974). Analysis of the sodium borohydride reduced tendon collagen demonstrated a major labelled amino acid eluting near to the position of glycine, thereby indirectly confirming the identity of this component (Fig 12). There were other tritiated peaks seen on this elution profile, one of which co-eluted with hydroxylysine. This was also observed in the acid and base hydrolysates of reduced fetal bovine skin collagen but in lower amounts. This peak is likely to be ^{3}H-hydroxylysinonorleucine

Table 3. Stability of the ^{3}H-Hydroxynorleucine to Acid and Base Hydrolysis.

The sodium borohydride-reduced collagen was hydrolyzed in acid or base, at 110°C for various periods of time. Aliquots of the hydrolysates were applied to the amino acid analyzer, fractions collected and radioactivity measured. The results are expressed relative to the phenylalanine content of the hydrolysates to compensate for any variation in the amount of collagen that was analyzed.
Note these results demonstrate the stability of the reduced cross-link precursors. Note that the relative amounts of these labelled amino acids after acid and base hydrolysis are similar.

	ACID HYDROLYSIS			BASE HYDROLYSIS		
HYDROLYSIS (hours)	HNL+ClNL (dpm)	PHE (nmole)	dpm / nmole [a]	HNL (dpm)	PHE (nmole)	dpm / nmole [a]
22	4309	3.85	1102	1447	1.38	1052
44	4687	4.40	1052	1467	1.43	1027
66	4154	3.90	1065	N.D.	N.D.	N.D.

HNL - hydroxynorleucine; ClNL - chlornorleucine;
PHE - phenylalanine; N.D. - not determined;
dpm - disintegrations per minute;
a - single determinations.

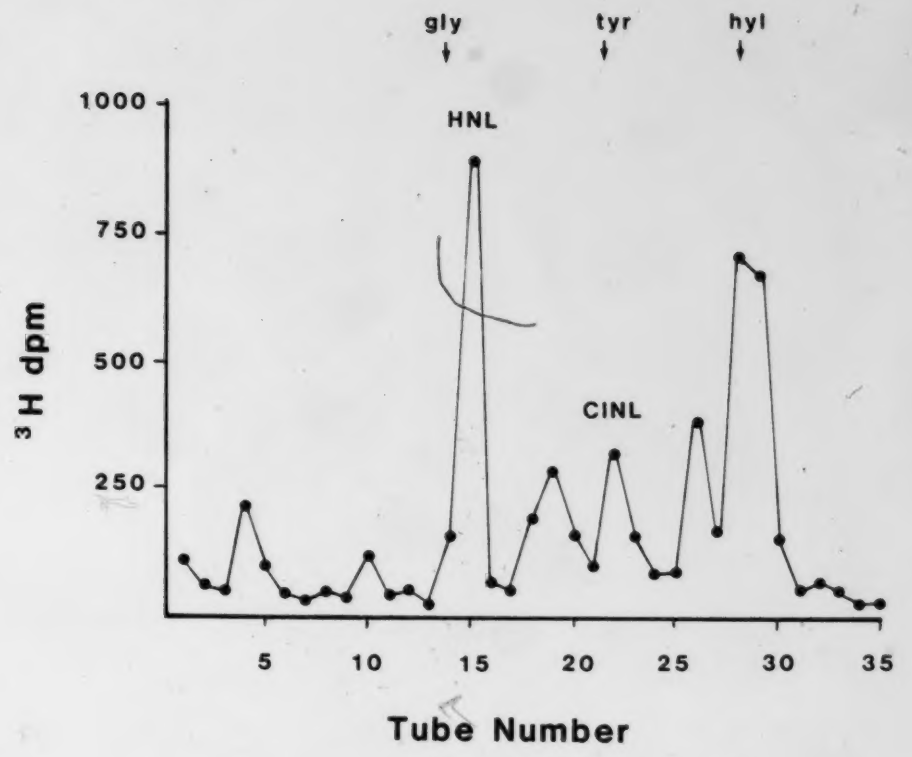


Figure 12. ^{3}H-Amino Acid Elution Profile of ^{3}H-Labelled Acid Soluble Rat Tail Tendon Collagen.

Acid soluble rat tail tendon collagen (35µg) was hydrolyzed in 5.7N-HCl, and one half of the sample applied to the amino acid analyzer. Fractions (0.5ml) were collected and radioactivity measured. (↓) elution positions of amino acids.
HNL - hydroxynorleucine; ClNL - chlornorleucine.

(Mechanic, 1974), which is indicative of the presence of some intermolecular cross-linking (aggregates) in the isolated acid soluble collagen from both tissues. However, since the unsaturated precursor of this labelled compound is labile in weak acids (see Fig 10), it was probably formed (or re-formed) during the purification but prior to reduction. In addition, the other labelled compounds shown in Fig 11 can be tentatively identified from the elution profile of Mechanic (1974): the peak eluting near to serine is likely to be dihydroxynorleucine and the peak eluting between hydroxynorleucine and chlornorleucine is probably the reduced aldol condensation product.

Aliquots of the reduced bovine collagen were hydrolyzed for different times in acid or base to determine the stability of the radiolabelled reduced cross-link precursors (Table 3). As shown these components appear to be relatively stable under the conditions of hydrolysis.

Action of Proteinases on ^{3}H-Collagen

An assay system was initially designed to detect the presence of proteolytic activity directed towards the telopeptide regions. Previously it had been described that at below denaturation temperatures and with limited incubation times and enzyme concentrations, the collagen, after incubation with conditioned medium, appeared to be degraded into the characteristic collagenase fragments. Gel filtration on a small column packed with material such as

Bio-Gel P10 (fractionation range 1,500-20,000) should effectively separate these collagenase fragments from any low molecular weight fragments (2,000 or less) that may be released by an activity directed towards the telopeptide regions.

The ^{3}H-collagen was incubated with trypsin to determine whether it was still in a native conformation (Fig 13). Incubation with denatured collagen demonstrated the elution profile expected if multiple cleavages on the substrate was occurring. In a later study, a longer incubation time with native collagen (20 hours) also did not result in significant release of low molecular weight labelled fragments.

Cathepsin D, (an acid proteinase known to act as a C-terminal telopeptidase; Scott and Pearson, 1978a, 1981), was incubated with the ^{3}H-collagen. Chromatography of the products on a column (1.5 x 7cm) packed with Bio-Gel P10, demonstrated a clear separation of high and low molecular weight labelled peptides (Fig 14). Approximately 11% of the total starting radioactivity of the substrate was released after correction for the no-enzyme control, with the majority being cleaved within 3 hours, at an enzyme to substrate ratio of 1:40 (w/w).

A large scale digestion with cathepsin D followed by ^{3}H-amino acid analysis of the contents of both the Vo fraction and Vt fraction determined the minimum amount of hydroxynorleucine present at the C-terminal extra-helical

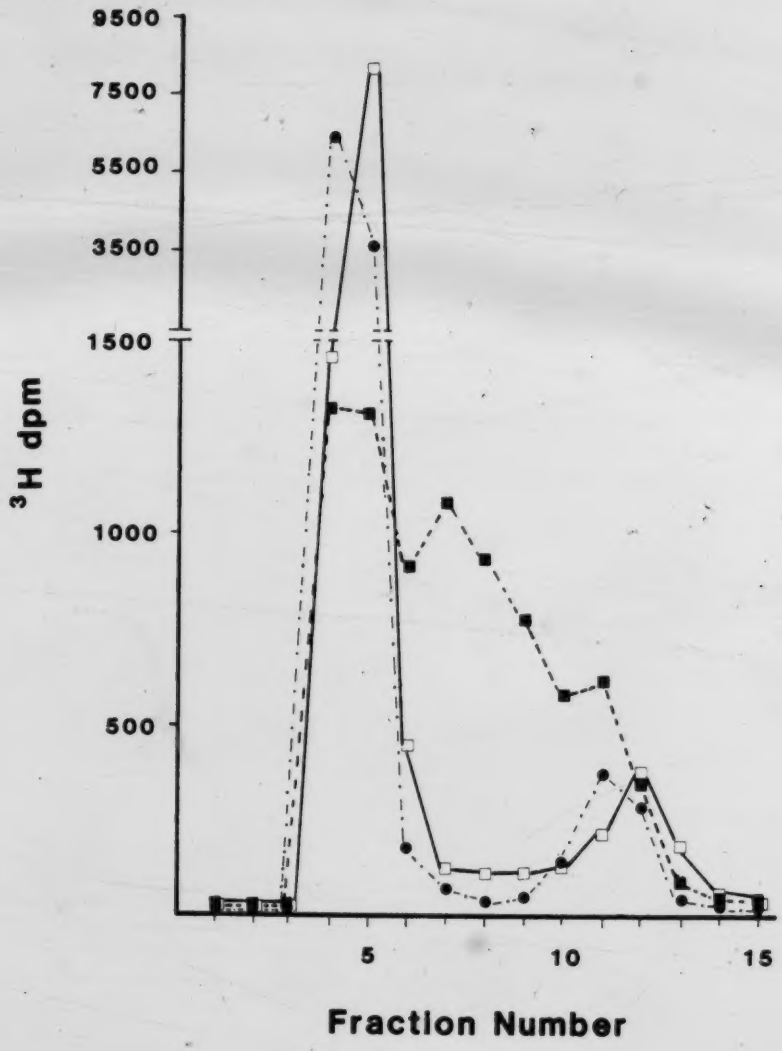


Figure 13. Elution Profile of Trypsin-Digested ^{3}H-Collagen on Bio-Gel P10.

Trypsin (0.3µg) was incubated with the reduced collagen (20µg) in assay buffer (final volume of 1.0ml) for 2 hours at 24°C. The reaction was terminated by the addition of soybean trypsin inhibitor (3µg). The products were fractionated on a column (1.5 x 7cm) packed with Bio-Gel P10, in 1.0N-formic acid. Fractions (0.9ml) were collected and radioactivity determined.
(●) no enzyme control.
(□) trypsin incubated with native collagen.
(■) trypsin incubated with denatured collagen (denatured by heating to 55°C for 20 minutes).

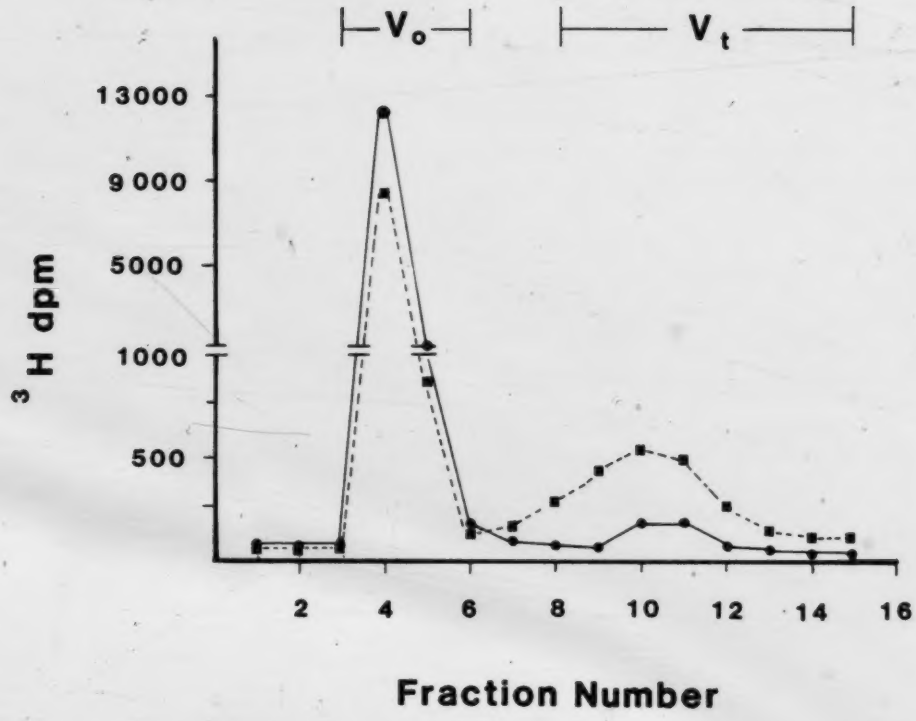


Figure 14. Elution Profile of Cathepsin D Digested ^{3}H-Collagen on Bio-Gel P10.

Tritiated collagen (20µg) was incubated with cathepsin D (0.5µg) in 0.14M-sodium acetate buffer pH 3.9, containing 0.02% sodium azide (final volume of 100µl), for 18 hours at 22°C. The reaction was terminated by the addition of pepstatin (0.43µg), the products were chromatographed, fractions (1.0ml) collected and radioactivity measured.
(●) no enzyme control.
(■) cathepsin D.
Note for purposes of quantitation the chromatogram was divided into 2 regions as shown. The radioactivity eluting in the Vt fraction after correction for control, was representative of an activity directed towards the telopeptide regions (as described in text).

region. The ^{3}H-amino acid elution profiles of the control and Vo and Vt fractions are shown in Fig 15. There was a decrease of ^{3}H-hydroxynorleucine (and ^{3}H-chlornorleucine) in the Vo fraction of the digested material as compared to the control, and appearance of these labelled amino acids in the Vt fraction. A no-enzyme control which was incubated and fractionated as described for the test substrate was shown not to have any acid-stable radioactive label in the Vt fraction. Both acid and base hydrolysates were analyzed so that the amounts of ^{3}H-hydroxynorleucine (and ^{3}H-chlornorleucine) could be related to acid or base stable amino acids. As shown in Table 4, approximately 64-68% of the total ^{3}H-hydroxynorleucine was removed by the action of cathepsin D when related to an acid or base stable amino acid. In addition, as expected, there were essentially background levels of hydroxyproline present in the Vt fraction which also confirms that cleavage had occurred only in the extra-helical region.

The amino-terminal telopeptide has also been described to contain modified lysine or hydroxylysine residues which have been demonstrated to take part in both intramolecular and intermolecular cross-links in fetal bovine skin collagen (see Introduction). To determine whether there were labelled amino acids at the N-terminal extra-helical region, the substrate was incubated with pepsin. This proteinase has been shown to cleave the N-terminal telopeptide proximal to the cross-link (reviewed by Burleigh, 1977), as well as

Figure 15. ^{3}H-Amino Acid Elution Profiles of Cathepsin D-Digested Collagen.

^{3}H-collagen (500µg) was incubated with cathepsin D (12.5µg), in 0.14M-sodium acetate pH 3.9, containing sodium azide (0.02%) in final volume of 1.5ml, for 25 hours at 18°C. The collagen digest was applied to a column (1.5 x 7cm) packed with Bio-Gel P10 and eluted in 1.0N-formic acid. Those fractions (1.0ml) corresponding to the low molecular weight peptides (Vt fraction; see Figure 14), were pooled, lyophilized, reconstituted in 1.0ml of 1.0N-formic acid, and rechromatographed. The fractions containing the low molecular weight peptides from the second chromatograph, as well, as the Vo fraction of the first chromatograph, were lyophilized, and an aliquot from each hydrolyzed in acid and base. The hydrolysates were examined on the amino acid analyzer as previously described.

a,b,c - samples hydrolyzed in 2.0N-NaOH.
d,e,f - samples hydrolyzed in 5.7N-HCl.

a,d - no-enzyme control (Vo fraction).
b,e - cathepsin D digested collagen (Vo fraction).
c,f - cathepsin D digested collagen (Vt fraction).

HNL - hydroxynorleucine; ClNL - chlornorleucine;

Note the decreased level of hydroxynorleucine (and chlornorleucine) in the Vo fraction of the cathepsin D digested collagen, and the appearance of these reduced cross-link precursors in the Vt fraction. The Vt fraction of the no-enzyme control (not shown) contained no acid or base stable labelled molecules.

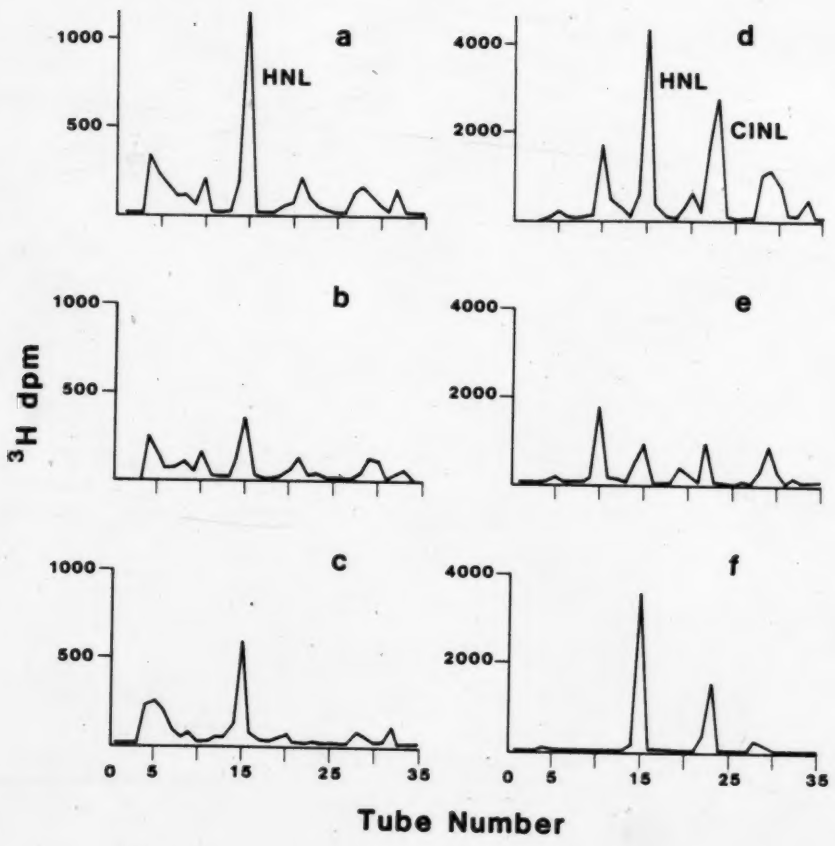

a
HNL
1000
500
d
HNL
CINL
4000
2000
b
1000
500
e
4000
2000
^{3}H dpm
c
1000
500
0 5 15 25 35
f
4000
2000
0 5 15 25 35
Tube Number

Table 4. Release of Low Molecular Weight Fragments Containing ^{3}H-Hydroxynorleucine from the Incubation of ^{3}H-Collagen and Cathepsin D.

The details of the experiment are as described in legend of Figure 15. The amount of radioactivity that was due to the reduced cross-link precursor was expressed as a function of an acid or base stable amino acid.

		ACID HYDROLYSATES		BASE HYDROLYSATES	
ENZYME PREPARATION	FRACT.	TOTAL[a] dpm	dpm / nmole(HYP)	TOTAL[b] dpm	dpm / nmole(PHE)
none	Vo	9234	240	1238	891
none	Vt	0	0	0	0
Cathepsin D	Vo	2673	77.5	375	319
Cathepsin D	Vt	5586	N.D.[c]	772	N.D.[c]
Released (%)			67.7		64.2

none - no-enzyme control; N.D. - not determined;
HYP - hydroxyproline; PHE - phenylalanine.
a - sum of dpm associated with hydroxynorleucine and chlornorleucine (Fig 15); single determinations.
b - dpm associated with hydroxynorleucine peak (Fig 15).
c - neither phenyalanine nor hydroxyproline present in carboxy-terminal peptide region.

acting at the C-terminal extra-helical region proximal to the cross-link site (Zimmerman et al., 1973). Following the same protocol as described above for cathepsin D, pepsin at an enzyme to substrate ratio of 1:20, released approximately 14-15% of the total starting radioactivity. The ^{3}H-amino acid analysis of the fractionated products from the large scale digestion (pepsin:collagen 1:25, in 0.017M-acetic acid/HCl, pH 1.9, for a 40 hour incubation, at 18°C), demonstrated similar results to those from the cathepsin D digestion. Only background levels of hydroxyproline were found in the Vt fraction, confirming that the substrate was native. The ^{3}H-amino acid elution profile (both in position of the peaks, and in the relative amounts of the individual peaks) of this fraction was not significantly different from that shown for cathepsin D in Fig 15. These results would suggest that either the content of labelled amino acids at the N-terminus was low, or that pepsin was ineffective in releasing it. In a study carried out at a later date, exhaustive digestion of acid soluble collagen with pepsin resulted in nearly complete (95% or greater) shortening of the α_1CB6, however action at the N-terminal telopeptide resulted in only an apparent decrease of 40-50% in the proportion of intramolecularly cross-linked collagen β chains (see Fig 29b).

Telopeptidase Activity in Conditioned Medium

Concentrated conditioned medium was incubated with the reduced collagen to determine whether it contained a proteolytic activity directed towards the telopeptide regions (Table 5). There appeared to be a correlation between increasing amounts of conditioned medium used in the incubations and the level of radioactivity recovered in the Vt fractions. From Table 5, and from other data the maximum level of total radioactivity released appeared to be quite similar to that demonstrated by pepsin (15%). In addition, the telopeptidase activity appeared to be effectively inhibited by the addition of EDTA.

In order to further characterize the low molecular weight peptides released by the proteolytic action of conditioned medium, a large scale incubation was performed. However, because the constituents of the standard assay buffer (Tris, NaCl) used for the small scale incubations would interfere with the amino acid analysis of the fractions (primarily the Vt fraction) another buffering system had to be used. Ammonium propionate (0.039M), pH 7.2, containing 0.005M-$CaCl_2$, and 0.02% (w/v) sodium azide but not containing NaCl, was selected for this purpose.

The results of the large scale digestion of the ^{3}H-collagen with conditioned medium are shown in Fig 16 and Table 6. This incubation was performed under conditions that should not have caused exhaustive digestion of the collagen (20 hour incubation at 18-20°C). An aliquot (100µl) of the

Table 5. Incubation of ^{3}H-Collagen with Conditioned Medium.

Conditioned medium was concentrated (61 fold) by ultrafiltration on Amicon PM 10 membranes. Activation was by treatment with soluble trypsin. The medium was incubated with the substrate (20µg; 15,000dpm) in assay buffer (final volume 200µl) for 22 hours at 22°C. Analyses were carried out as described in Figure 14. Values are results of single determinations.

ENZYME PREPARATION	VOLUME (µl)	Vo(dpm)	Vt(dpm)	CORRECTED Vt (dpm)a	Vt TOTAL(%)b
no-enzyme	0	5334	928	0	0
medium	20	6072	987	59	0.4
medium	50	5167	1192	264	1.8
medium	100	7080	1649	721	4.8
no-enzyme	0	5729	537	0	0
medium (act.)	20	7625	1292	755	5.0
medium (act.)	50	5557	2324	1787	11.9
medium (act.)	100	3421	2934	2397	15.9
no-enzyme	0	5334	928	0	0
medium (act.) +EDTA (0.05M)	50	8587	992	64	0.4

act. - activated.
a - corrected for no-enzyme control values in dpm.
b - corrected dpm in the Vt fraction as a percent of the total dpm (15,000).

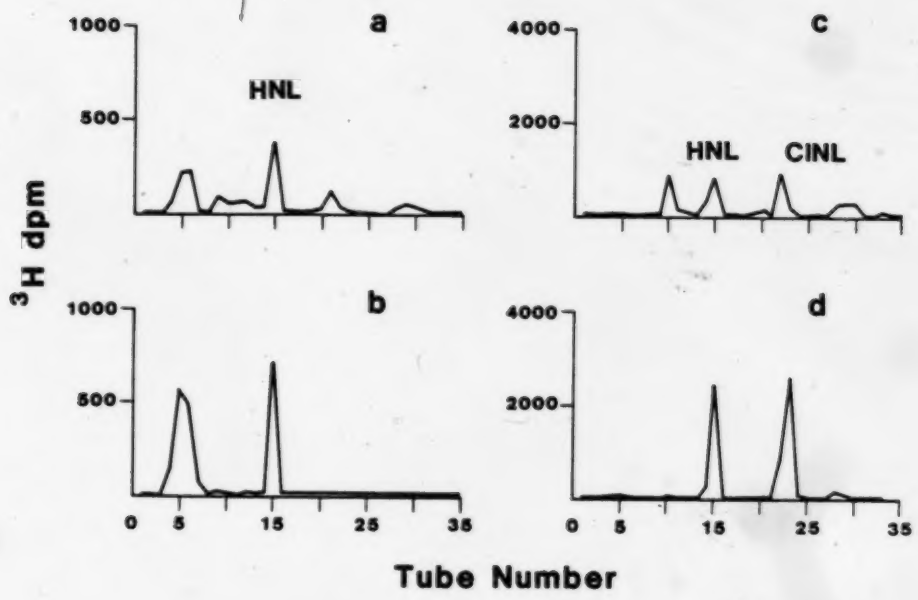


Figure 16. ^{3}H-Amino Acid Elution Profiles of the Fractionated Products of the Incubation of ^{3}H-Collagen and Conditioned Medium.

Conditioned medium (concentrated 60 fold) was dialyzed against 0.039M-ammonium propionate buffer, pH 7.4, (containing 0.005M-calcium chloride, and 0.02% sodium azide) overnight. The medium (0.8ml) was treated with trypsin in solution (6µg) for 15 minutes at 22°C, followed by the addition of soybean trypsin inhibitor (40µg). The incubation mixture contained 500µg of collagen with activated medium, in final volume of 2.6ml of buffer, pH 7.4. After 20 hours, at 18°C, the collagen digest was fractionated into Vo and Vt fractions. The Vt fraction was rechromatographed (see Figure 15), and aliquots of the 2 fractions, hydrolyzed in acid or base, and applied to the amino acid analyzer. Fractions (0.5ml) were collected and radioactivity measured.

a,b - base hydrolysis.
c,d - acid hydrolysis.
a,c - the Vo fraction.
b,d - the Vt fraction.

HNL - hydroxynorleucine; ClNL - chlornorleucine. Note the appearance of a significant amount of the labelled cross-link precursor in the Vt fraction.

Table 6. Digestion of ^{3}H-Collagen by Conditioned Medium; Analysis of the Amount of ^{3}H-Hydroxynorleucine in the Hydrolysates of the Bio-Gel P10 Fractions.

^{3}H-Collagen was incubated with conditioned medium as described in Figure 16. The level of ^{3}H-hydroxynorleucine (and ^{3}H-chlornorleucine of acid hydrolysates) in the fractions from Bio-Gel P10 chromatography were expressed as function of an acid or base stable amino acid. Values are results of single determinations.

		ACID HYDROLYSATES		BASE HYDROLYSATES	
ENZYME PREPARATION	FRACT.[a]	TOTAL[b] dpm	TOTAL dpm / nmole(HYP)	TOTAL[c] dpm	dpm / nmole(PHE)
no-enzyme	Vo	9234	240	1238	891
no-enzyme	Vt	0	0	0	0
medium	Vo	2364	103	435	N.D.[d]
medium	Vt	6097	N.D.[e]	744	N.D.[e]
% release of ^{3}H-HNL		52	57	63	-

a - fraction; referring to chromatography on Bio-Gel P10, see Figure 14.
b - ^{3}H-hydroxynorleucine and ^{3}H-chlornorleucine
c - ^{3}H-hydroxynorleucine in base hydrolysates
d - not determined, phenylalanine from medium protein interferes.
e - not determined, neither phenylalanine nor hydroxyproline present in carboxy-terminal peptide.

conditioned medium was hydrolyzed and analyzed for hydroxyproline on the amino acid analyzer. The level of hydroxyproline in the dialyzed conditioned medium that was incubated with the substrate was equivalent to 1% or less of the collagen used in the incubation. It is quite clear from these results that the ^{3}H-hydroxynorleucine was released by the action of a proteinase or proteinases from the conditioned medium. It was previously shown that at least two-thirds of the ^{3}H-hydroxynorleucine was located in the C-terminal extra-helical region (see Table 4; action of cathepsin D). Thus the release of 50-60% of this cross-link precursor on incubation with conditioned medium demonstrates that there is a proteolytic activity that can cleave at the C-terminal end of collagen proximal to the cross-link site. In addition, the absence of a significant amount of hydroxyproline in the Vt fraction strongly suggests that the cleavage had occurred between residues C1-C16 of the telopeptide.

Refinement of the Telopeptidase Assay

The system that was utilized in the earlier studies established the presence of an activity in conditioned medium directed towards the C-terminal telopeptide region of Type I collagen. It was observed that recoveries of the low molecular weight peptides in the Vt fraction appeared to be a consistent and reliable means of determining the overall affect of the proteinases on the tritiated collagen.

However, there were a number of problems associated with the chromatography of small amounts of collagen substrate (10-20μg) as discussed below. In addition, the substrate itself was not ideally labelled. Only 20% of the total label on the ^{3}H-collagen was stable towards acid or base.

The use of 1.0N-formic acid was previously established as allowing for the highest recoveries of collagen peptides (Scott, P.G., 1980). However, extended exposure of the column material (Bio-Gel P10) to 1N-formic acid, invariably resulted in decreased recoveries of the large molecular weight labelled peptides. Thus the column material was used for only one chromatograph and exposure to the formic acid was limited to 3 column volumes before application of sample. In addition, in the final method smaller columns (0.7 x 4cm; 2ml settled bed volume) were used to minimize losses caused by adsorption, with no apparent loss in resolution.

Significant losses of the large molecular weight peptides were still evident, presumably caused by binding of the charged collagen molecules to the Bio-Gel P10, or to the plastic columns. Addition of a carrier before gel filtration significantly improved recoveries. Bovine serum albumin (200μg) was found to be a more efficient carrier for this purpose than collagen (Table 7).

The temperature of incubation was previously shown to be quite important. There is a possibility that at 25°C a portion of the α1/4 peptides may assume a random coil

Table 7. Recovery of ^{3}H-Collagen on Bio-Gel P10 Chromatography; Effect of Protein Carriers.

Aliquots of ^{3}H-collagen (19µg, 14,000dpm) were dispensed into microfuge tubes. To each was added either cold acid soluble collagen or bovine serum albumin, or a combination of the two. The volumes were made up to 195µl with buffer, concentrated formic acid (12µl) was added, and the mixtures chromatographed on columns (0.7 x 4cm) packed with Bio-Gel P10. Fractions of: (1) 1.2ml; (2) 0.2ml; (3) 0.2ml; (4) 1.3ml; were collected and radioactivity measured. Values are results of single determinations in each case.

	dpm/FRACTION					
CARRIER	1(Vo)	2	3	4(Vt)	TOTAL (dpm)	RECOVERED (%)
none	5961	152	78	646	6837	48.0
collagen-100µg	8771	331	97	625	9824	69.5
albumin-200µg	10165	244	89	562	11060	78.3
collagen-100µg +albumin-200µg	6625	2499	861	1185	11170	79.1

note - the highest recoveries were obtained with the combination of albumin and collagen as carriers, however considerable trailing in the intermediate region (fractions 2 and 3) indicated that too much protein had been applied to the column.

configuration for long enough to be attacked by non-specific proteinases ('gelatinases') present in the conditioned medium. This could give rise to spuriously high counts in the Vt fraction, due to non-specific labelling on the collagen. Thus incubations were for the most part carried out at 18-20°C, to diminish this possibility. As noted previously, in the large scale ^{3}H-collagen incubation with conditioned medium, there was a negligible amount of hydroxyproline in the Vt fraction, which indirectly confirms that the substrate was not 'over-digested'.

Non-specific labelling of the substrate did not interfere with the analysis of the ^{3}H-labelled amino acids, presumably because this was lost as water during hydrolysis and subsequent drying. However in a quantitative assay the relatively high background levels due to the continual exchange of label with the surrounding solution will decrease the sensitivity. Exhaustive dialysis of the reduced, labelled collagen (after the initial dialysis against 0.01M-acetic acid) against 4.5% (v/v) ethanol, and 0.02M-sodium borate pH 9.1 caused losses of approximately 20% and 70% respectively of the total activity. The collagen dialyzed against the latter solution was determined by amino acid analysis to have the same ^{3}H-amino acid elution profile as the collagen that was not dialyzed against the sodium borate buffer. For later work, the collagen substrate was routinely treated with sodium borate after reduction.

D. Definition of Units of Activity

Telopeptidase Activity

While an activity directed towards the C-terminal extra-helical region had been demonstrated in conditioned medium, the ^{3}H-collagen probably had labelled amino acids at the N-terminal telopeptide region. Thus with this substrate, activity detected must be defined as telopeptidase activity rather than C-terminal telopeptidase activity.

A curve relating activity to amount of enzyme is shown in Fig 17. The telopeptidase preparation was one that was purified by affinity chromatography on immobilized collagen as described below ('Purification of the Neutral Metalloproteinases'). The collagen substrate that was used had a specific activity of 32dpm of ^{3}H-hydroxynorleucine/μg collagen. Obviously, from what was observed in Fig 17, the linear range of this assay is very small. This did prove to be a handicap in obtaining accurate values for units of activity. In later experiments, collagen was reduced with ^{3}H-sodium borohydride which had been diluted with less cold carrier, and thus had a higher specific activity; approximately 64dpm of ^{3}H-hydroxynorleucine/μg of collagen. Use of this collagen, as well as incubating double the amount of the substrate (20μg) increased the range of the assay and hence its precision.

The units of telopeptidase activity were defined as follows: One unit of telopeptidase activity will give

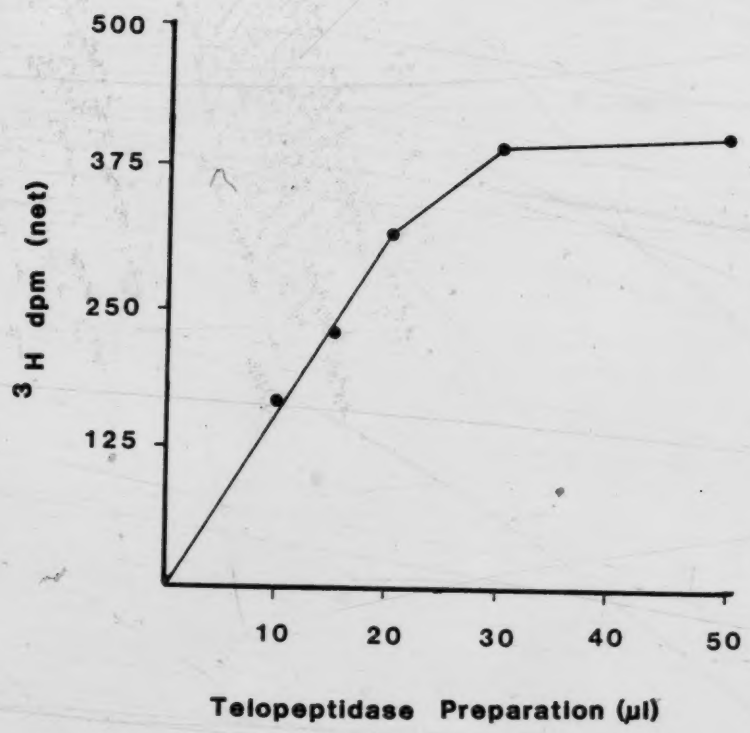


Figure 17. Incubation of ^{3}H-Collagen and an Enzyme Preparation containing Telopeptidase Activity; Effect of Varying Enzyme Concentration.

Varying amounts of a preparation of telopepeptidase activity that was purified by collagen-affinity chromatography, were incubated with ^{3}H-collagen (10µg/20µl) in assay buffer (final volume 90µl), for 4 hours at 18°C. The digests were fractionated into Vo and Vt fractions and radioactivity measured. The radioactivity associated in the Vt fractions after correction for the no-enzyme control with the different test solutions demonstrates a linear response. Values are results of single determinations in each case.

maximum release of the radioactivity associated with 1μg of collagen-telopeptide in 1 hour at 20°C. However, there is some non-specific (e.g. acid-labile) labelling associated with the C-terminal telopeptide region (approximately 20% of the total radioactivity that was removed by cathepsin D, or by proteolytic activity of conditioned medium). Thus in the collagen substrate used for the experiment shown in Fig 17, maximum release from 10μg of collagen was approximately 400dpm; therefore 1 unit was defined as that amount of activity releasing 40dpm/hour. With the other ^{3}H-collagen substrates used in studies conducted at a later stage, the linear range for release of radioactivity was demonstrated to be between 100 and 1400dpm for 20μg of collagen, and the maximum release of label was 1600dpm after correction for background. Thus one unit of telopeptidase activity would release 80dpm/hour.

Collagenase

Collagenase activity was demonstrated using the technique involving collagen gels (reconstituted collagen) in capillary tubes. This method was quite sensitive in that 5μl of unconcentrated conditioned medium gave a measurable response in a 24 hour incubation. The effect of varying amounts of collagenase activity was studied using concentrated conditioned medium (Fig 18). The rate of digestion was linear only within the limits of 1-4mm clearance of the collagen gel in a 21 hour incubation.

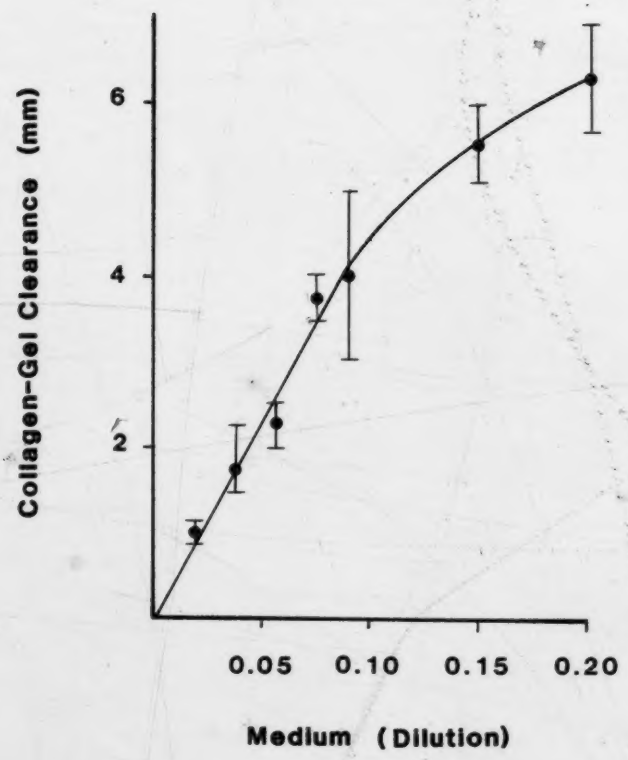


Figure 18. Incubation of Reconstituted Collagen with Varying Amounts of Conditioned Medium.

Concentrated (25 fold) conditioned medium was treated with trypsin-agarose, and aliquots (10µl) were dispensed into micro-centrifuge tubes. Varying amounts of assay buffer were added to each to give the appropriate dilution. Aliquots (5µl) were applied (in duplicate) to collagen gels in capillary tubes, and the level of clearance measured after incubation for 21 hours at 35°C. Note the linear response between 1-4mm of clearance. The points shown are mean values of duplicate determinations with the ranges.

However the rate of digestion appeared to be unaltered over 118 hours for those incubations containing low levels of collagenase activity (Fig 19). With higher concentrations of conditioned medium, the rate of clearance of the collagen gels appeared to show a biphasic response. The unit of collagenase activity was defined as that amount of activity causing the degradation of 1μg of collagen (equivalent to the clearance of 1.2mm of collagen gel) in one hour at 35°C. Thus the clearance of 4mm of collagen in 21 hours would be equivalent to approximately 0.16 units or 32 units per ml of enzyme solution.

A test of the stability of the collagen gels towards non-specific proteinase activity was made with trypsin (1μg). After 24 hours at 35°, there was no apparent clearance of the collagen gel. The trypsin solution was removed by aspiration and incubated with Azocoll overnight. Solubilization of the Azocoll occurred, indicating that at least a portion of the trypsin retained activity over this period but was ineffective against the collagen gel.

In addition to the above assay, an attempt was made to develop an assay using radiolabelled collagen. Acid soluble collagen was labelled with ^{14}C-acetic anhydride in which it was calculated that on average 1.4 residues of lysine or hydroxylysine per α chain were modified with the radioactive label (750dpm/μg collagen). This collagen was not denatured during the modification procedure as demonstrated by the apparently minimal effect of trypsin (Fig 20).

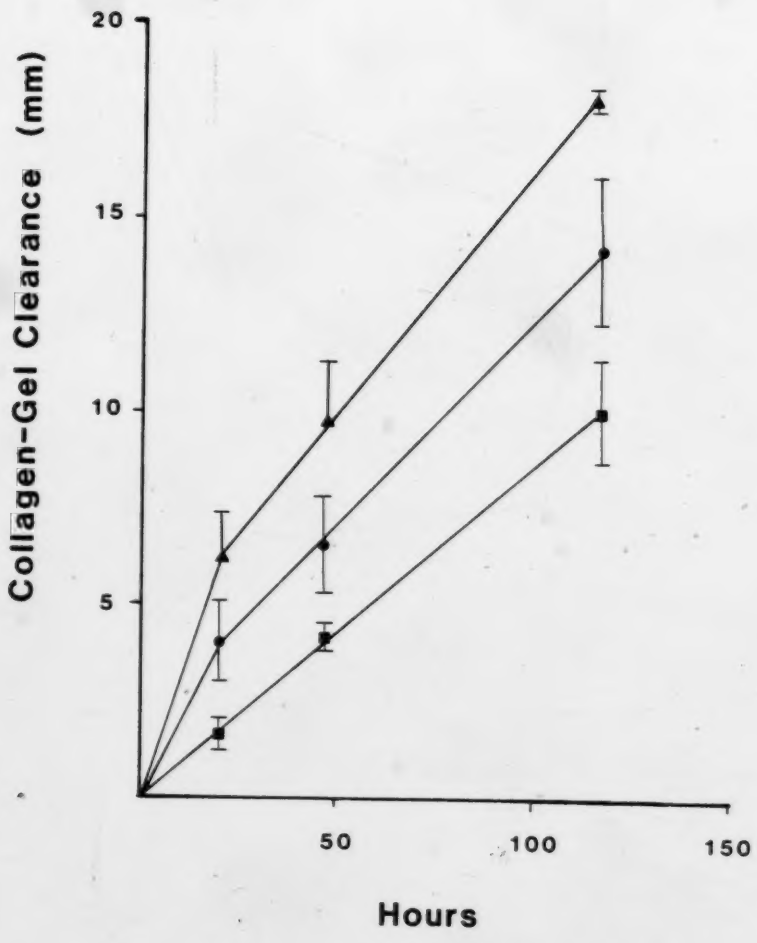


Figure 19. Incubation of Reconstituted Collagen with Conditioned Medium; Time Study.

Varying concentrations of conditioned medium were incubated with the collagen-gels in capillary tubes (see Figure 18 for details). The level of clearance of the collagen-gels was measured at the times noted on figure.
(■) 0.039 relative concentration of conditioned medium.
(●) 0.09 relative concentration.
(▲) 0.20 relative concentration.
The points shown are mean values of duplicate determinations with the ranges.

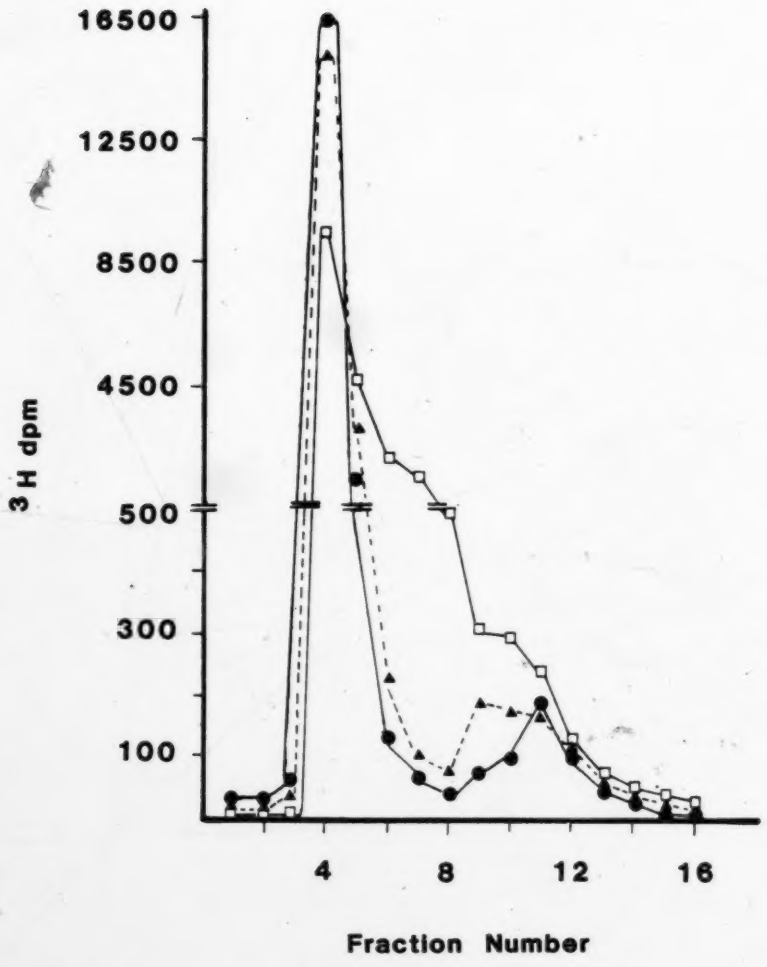


Figure 20. Elution Profiles of Products of Incubation of ^{14}C-Collagen and Trypsin.

Native and denatured (60°C for 20 minutes) ^{14}C-acetylated collagen (20µg; 27,000dpm) were incubated with TPCK-trypsin (2µg) in assay buffer (final volume 70µl) for 2 hours at 22°C. The reactions were stopped by the addition of soybean trypsin inhibitor (10µg). The products were chromatographed on columns (1.5 x 7cm) packed with Bio-Gel P10. Fractions (0.9ml) were collected and radioactivity measured.

(●) control; native collagen with premixed soybean trypsin inhibitor and trypsin.
(▲) native collagen with trypsin.
(□) denatured collagen with trypsin.

Note the control and the trypsin digested collagen gave similar profiles.

Several different methods for the determination of collagenase activity using this radiolabelled substrate were attempted, however all without success (Table 8). In general, background levels of radioactivity were unacceptably high, up to 50%, so that it was not possible to obtain reliable data. Chromatography of the collagen digests on a variety of materials packed in small columns (1.5 x 7cm) in order to isolate the α1/4 peptides from the other larger digested and undigested peptides, also was without success.

In the latter part of this investigation a standard curve for collagenase action was defined using the electrophoresis of the digested collagen peptides on 5% polyacrylamide gels. The collagenase preparation (Fraction B) was obtained by gel chromatography (agarose A0.5M) of the conditioned medium as described below ('Purification of Neutral Metalloproteinases'). The areas of the band corresponding to $\alpha_1$3/4 peptide were estimated from the scans and compared to the undigested α_1 chains (Fig 21,22). While this technique was not a particularly accurate method for determining collagenase activity, due to the inherent problems of quantitation of the Coomassie Blue-stained bands, as well as being quite time-consuming, some useful data was nevertheless obtained as described in a later section. One unit of collagenase was defined for this assay as that activity giving digestion of 1μg of collagen substrate per hour at 22°C.

Table 8. Collagenase Assays Attempted with the ^{14}C-labelled Collagen.

a) Terato et al. (1976): which involved incubating collagen (in solution) at 37°C, and the quantitation of the amount of collagen peptides that were not precipitated by 50% dioxane.

b) Nagai et al. (1966): which involved the incubation of a collagen gel (reconstituted collagen), and the measurements of the amount of collagen that was solubilized.

c) Fractionation of the collagenase-derived products by gel filtration chromatography for the measurements of α 1/4 peptides. Due to the large numbers of samples to be analyzed, small columns packed with Ultragel AcA34, or Sephadex G-200 (fine or superfine), were used. The separation that was required for accurate analysis of collagenase activity could not be obtained.

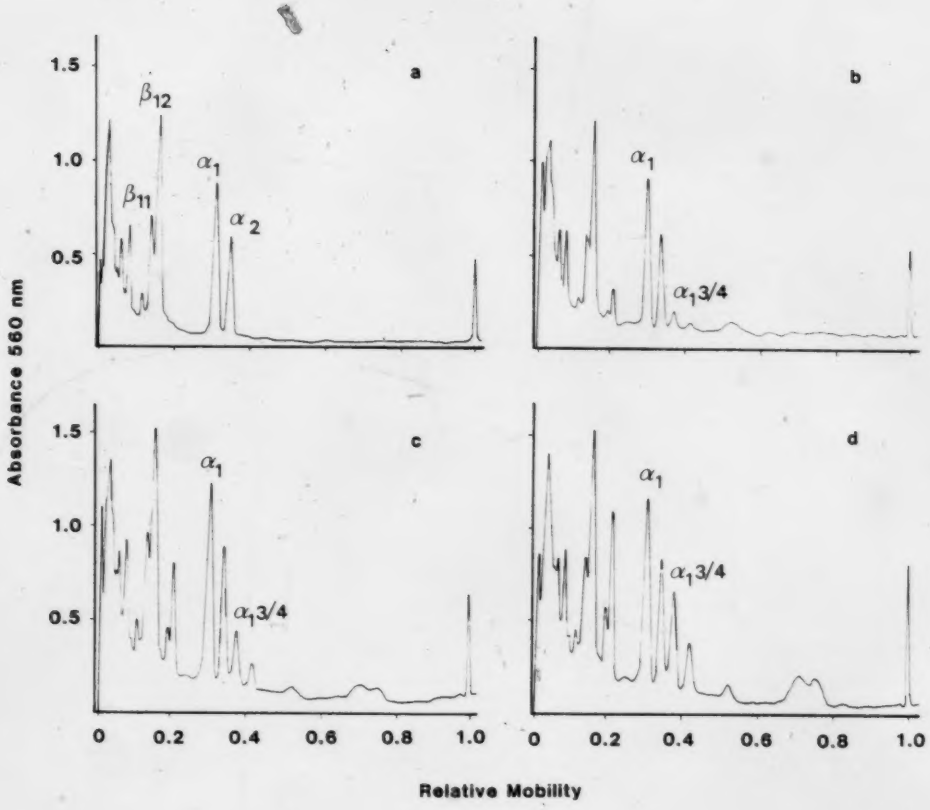


Figure 21. Gel Electrophoresis of Acid Soluble Collagen Digested by Fraction B; Development of a Collagenase Assay.

Acid soluble collagen (150µg) was incubated with varying amounts of a partially purified preparation of collagenase (Fraction B; see Figure 24), in assay buffer (final volume of 1.35ml), for 1 hour at 22°C. The reaction was stopped by addition of EDTA to final concentration of 0.05M. Following dialysis against water, the products were electrophoresed on 5% polyacrylamide SDS gels.

a) no-enzyme control.
b) 10µl of Fraction B.
c) 20µl of Fraction B.
d) 40µl of Fraction B.
The level of conversion of α_1 to $\alpha_1$3/4 as described in Methods, is shown in Figure 22.

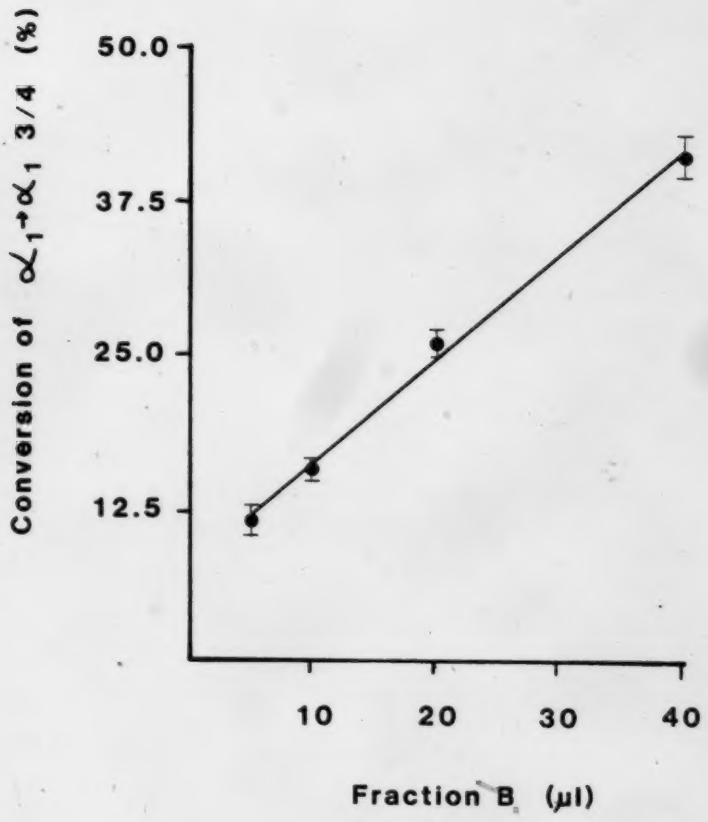


Figure 22. Acid Soluble Collagen Digestion by Collagenase; Effect of Varying Enzyme Concentrations.

Acid soluble collagen was incubated with varying amounts of Fraction B and the products electrophoresed on 5% polyacrylamide gels (see Figure 21 for details). The conversion of α_1 to α_1 3/4 was quantitated, as described in Methods, and plotted according to added enzyme as shown. The values are of duplicate determinations (gel scans) of single incubations, plotted with the ranges.

For comparison purposes, it was estimated that one unit of collagenase activity on the collagen gel capillary method was equivalent to approximately 10 units of activity using the soluble collagen electrophoresis technique.

E. Concentration and Activation of Medium

In the earlier investigations, conditioned medium was concentrated by ultrafiltration and activated with soluble trypsin. In the later studies, conditioned medium was concentrated by stepwise precipitation with ammonium sulphate. Test of recoveries of telopeptidase activity with varying pH demonstrated that it was not necessary to adjust the pH of the conditioned medium prior to addition of the ammonium sulphate (Table 9). The majority of the recoverable telopeptidase activity (80% or better; trypsin activated) appeared to be precipitated in the 20-60% saturated ammonium sulphate step. Although not shown, the activity in the 20% saturated ammonium sulphate precipitate was always negligible (1% or less of original). In a separate experiment, the recovery of Azocoll-digesting activity in the various fractions was demonstrated to be similar to that above. Approximately 70% of the total activity (after activation) was in the 60% ammonium sulphate precipitate with the remainder in the 90% supernatant. Less than 1% of the activity was in the 20% ammonium sulphate precipitate.

In the earlier studies, the latent proteinases of conditioned medium were activated by treatment with soluble

Table 9. Recovery of Telopeptidase Activity from Conditioned Medium by Ammonium Sulphate Precipitation; Effect of pH.

Aliquots (25ml) of conditioned medium were adjusted to the desired pH by the addition of Tris/HCl (final concentration of 0.05M), for the tests at pH 7 and pH 8, and with Tris/Maleate (0.05M) for the test at pH 6. The control was unadjusted. The pH of the test solutions shown below, are the pH of the mixtures containing 60% saturated ammonium sulphate. The results are expressed as total units of telopeptidase recovered at each step. The 20% saturated ammonium sulphate step contained negligible activity. The precipitated protein was dissolved and dialyzed against assay buffer, and solutions treated with trypsin-agarose (10 minutes). The values are the means of duplicate determinations with the ranges.

STEP	CONTROL[a]	pH 6.3	pH 7.3	pH 7.9
60%	37.2±4.1	42.6±2.5	37.4±3.3	44.0±4.4
90%	9.6±1.9	8.8±1.7	7.2±0.1	7.6±1.0
total	46.8±6.0	51.4±4.2	44.6±3.4	51.6±5.4

a - pH not adjusted, but was approximately pH 7.0.

trypsin. To inhibit the trypsin activity, a 5-10 fold molar excess of soybean trypsin inhibitor was added. For the purification of proteinases, the inclusion of large amounts of protein material was not desirable. Also, the addition of the soybean trypsin inhibitor could possibly have inhibited an activity of interest. Concentrated conditioned medium was treated with trypsin in solution or with trypsin-agarose and then tested for the ability to solubilize Azocoll. There was essentially no difference between these results. In a different experiment there was also no apparent difference in the telopeptidase activity.

In a later study another activator, p-hydroxymercuri-benzoate, was used. Also, in this case there was no significant difference in the level of active telopeptidase activity compared to those obtained using trypsin-agarose.

F. Purification of the Neutral Metalloproteinases

Affinity Chromatography

The initial attempts at purification of the proteinases involved affinity chromatography as a first step. Concentrated conditioned medium that had been dialyzed against assay buffer containing no sodium chloride was chromatographed on a column (1.5 x 2.5cm) packed with CNBr activated Sepharose 4B to which acid soluble collagen with intact C-terminal telopeptide had been bound. Results of two of the initial experiments are shown in Table 10. It would

Table 10 Affinity Chromatography of Conditioned Medium on Immobilized Collagen.

Concentrated (25-fold) conditioned medium (2.5ml and 3.8ml respectively) was dialyzed against assay buffer containing no sodium chloride, and applied to columns(1.5 x 2.5cm)packed with collagen bound to cyanogen bromide activated Sepharose 4B. The columns were washed with approximately 10 column volumes each of assay buffer containing no sodium chloride and 1.0M-NaCl. Fractions were analyzed for the activities as described below. The levels of activity in each step are expressed as a percentage of the total activity recovered. Values are results of single determinations.

ACTIVITIES	NON-RETARDED (0.0M-NaCl) % of TOTAL	RETARDED (1.0M-NaCl) % of TOTAL
EXP'T 1		
azocoll digesting	73	27
collagenase	54	46
telopeptidase	N.D.	N.D.
EXP'T 2		
azocoll digesting	68	32
collagenase	52	48
telopeptidase	36	64

N.D. - not determined

appear that a portion of both the collagenase and telopeptidase activities bound to the column material and were partially separated from the majority of the Azocoll-digesting activity.

The CNBr activated Sepharose 4B that was used initially was a preparation that was 2 years past the expiry date. Chromatography of conditioned medium on a fresh batch of CNBr activated Sepharose 4B (with bound collagen) resulted in essentially no binding of the proteolytic activity. The chromatography was repeated, with the same result. It was thought possible that the collagen was bound at too many sites on the activated Sepharose, thereby limiting the access of the proteinases. An alternate explanation is that other macromolecules in the conditioned medium may compete with the proteinases for a limited number of binding sites on the collagen.

Another technical problem encountered was in the neutralization of the concentrated collagen solution prior to coupling. Using the recommended 'coupling' buffers (sodium bicarbonate containing 0.2M-NaCl, pH 8.5) as was described by Bauer et al. (1971), frequently caused the collagen to precipitate. This was overcome by using sodium citrate buffer at pH 6.5. The collagen remained in solution and fairly efficent binding (50% of the added collagen) was obtained.

Chromatography of conditioned medium on this preparation of immobilized collagen, apparently separated a

significant proportion of the telopeptidase activity from the collagenase activity (Table 11). The majority of the collagenase activity however, was lost, probably during dialysis. Loss of proteolytic activity on dialysis against buffer solutions of low salt is a problem that has been documented in the literature (see Discussion). According to the results shown in Table 11, approximately 50% of the detected telopeptidase activity was apparently recovered in the 0.6M-NaCl wash, while most of the Azocoll digesting and collagenase activities were eluted earlier. No collagenase activity was found in the 0.6M or 1.0M-NaCl fractions, even after 1 week of incubation (collagen-gel capillary assay). The above results could not be reproduced. Chromatography of a different preparation of conditioned medium on the same column material as well as on a new preparation of collagen-Sepharose, resulted in essentially no retardation of proteolytic activity. Further attempts using the affinity system were made in later studies, however the basic problems were not solved.

Telopeptidase Activity from Collagen-Affinity Chromatography

Due to the apparent separation of a telopeptidase activity from all active collagenase in the experiment as described above (Table 11), some characteristics of this proteinase were examined. With this preparation a standard curve of activity was prepared (Fig 17), and units of activity were established. Various inhibitors of proteolytic

Table 11. Affinity Chromatography of Conditioned Medium on Immobilized Collagen; Isolation of a Telopeptidase Activity.

Conditioned medium that was dialyzed against assay buffer containing no sodium chloride was activated and applied to a column packed with collagen-Sepharose 4B. Approximately 5-8 column volumes of buffer containing the various concentrations of NaCl (see below) were applied, and the level of various activities measured. The level of proteolytic activity in each step of the chromatogram is expressed as a percentage of the total recovered activity. Values are results of single determinations.

	RECOVERED (%) of TOTAL			
ACTIVITIES/NaCl CONC.	0.0M	0.2M	0.6M	1.0M
azocoll digesting	58	25	12	4
collagenase	39	61	0	0
telopeptidase	26	25	49	<1

CONC. - concentration;

enzymes were tested. Incubation with EDTA (0.05M) caused the loss of at least 80% of the activity, while NEM (0.0017M), PMSF (0.0017M) and soybean trypsin inhibitor (100μg/ml) had little or no effect. An aliquot of this proteinase preparation was chromatographed on a column (1 x 115cm) packed with agarose A0.5M. The telopeptidase activity elution profile (not shown) demonstrated that the majority (90%) of the activity was eluted with an apparent Mr of 25,000-35,000. In addition, there was apparently a trace amount of activity eluting with an apparent Mr of 70,000. The last aliquot of this proteinase preparation was lost during a second gel filtration run, thus confirmation of the apparent Mr was not obtained.

Gel Filtration Chromatography of Conditioned Medium

Conditioned medium (2ml) was activated and chromatographed on a column (1 x 115cm) packed with agarose A0.5M (Fig 23). The majority of the telopeptidase had an apparent Mr of 70,000. While collagenase was shown in the fractions of an apparent Mr of 35,000-45,000, there was no apparent collagenase activity in the fractions of Mr of 70,000 demonstrable on the collagen-gels in capillary tubes in a 48 hour incubation.

The proteolytic activities (collagenase and telopeptidase) that were precipitated in the 60% and 90% saturated ammonium sulphate were chromatographed on a gel filtration column after each were dialyzed against the

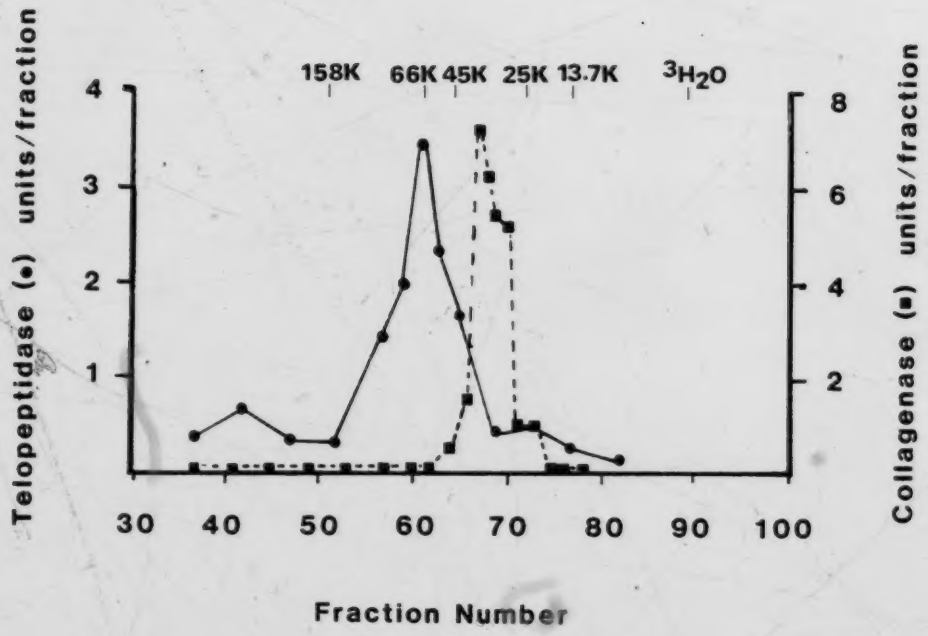


Figure 23. Elution Profiles of Proteolytic Activity of Conditioned Medium on Agarose A0.5M.

Activated (trypsin-agarose) conditioned medium (2.0ml) was applied to a column (1 x 115cm) packed with agarose A0.5M, which was equilibrated in chromatography buffer (0.05M-Tris/HCl, pH 7.4, containing 0.2M-NaCl, with 0.005M-$CaCl_2$, 0.05% Brij 35, 0.03% toluene), and eluted in this buffer at 4ml per hour. Fractions (1.04ml) were collected and aliquots assayed for telopeptidase activity and collagenase activity (collagen gels in capillary tubes). Shown are the relative elution positions of standard proteins and the tritiated water.

158K - aldolase; 66K - bovine serum albumin;
45K - ovalbumin; 25K - chymotrypsinogen;
13.7K - ribonuclease;

Note the telopeptidase activity had a relative elution consistent with an apparent molecular weight of approximately 70,000 daltons.

chromatography buffer and treated with trypsin-agarose (results not shown). There were no major differences in the elution profiles of the collagenase and telopeptidase activities between the two proteinase preparations (60% and 90% ammonium sulphate precipitates).

Conditioned medium (1.2 litre) was processed as described in the Methods section following the protocol of Cawston and Tyler (1979) for the purification of collagenase. The level of telopeptidase activity after treatment with pHMB was not significantly different from that resulting from treatment with trypsin-agarose (within 5%). One half of the concentrated medium was chromatographed on a column (2.5 x 115cm) packed with agarose A0.5M (Fig 24). As demonstrated, the elution profiles of the telopeptidase and collagenase activities were generally similar to that shown in Fig 23 for non-concentrated conditioned medium on a smaller scale. Telopeptidase activities from both fractions (Fraction A and Fraction B respectively, as labelled in Fig 24a) were inhibited (80-100%) by 0.03M-EDTA. Analysis of the Azocoll-digesting and azocaseinolytic activities are shown in Fig 24b. The apparent peaks of activities against these substrates, appear to coincide with the collagenase and telopeptidase activities of Fraction B. The second half of the concentrated medium was chromatographed and the fractions analyzed. The proteolytic activity elution profiles for both the collagenase and telopeptidase activities were similar to those shown in Fig 24a.

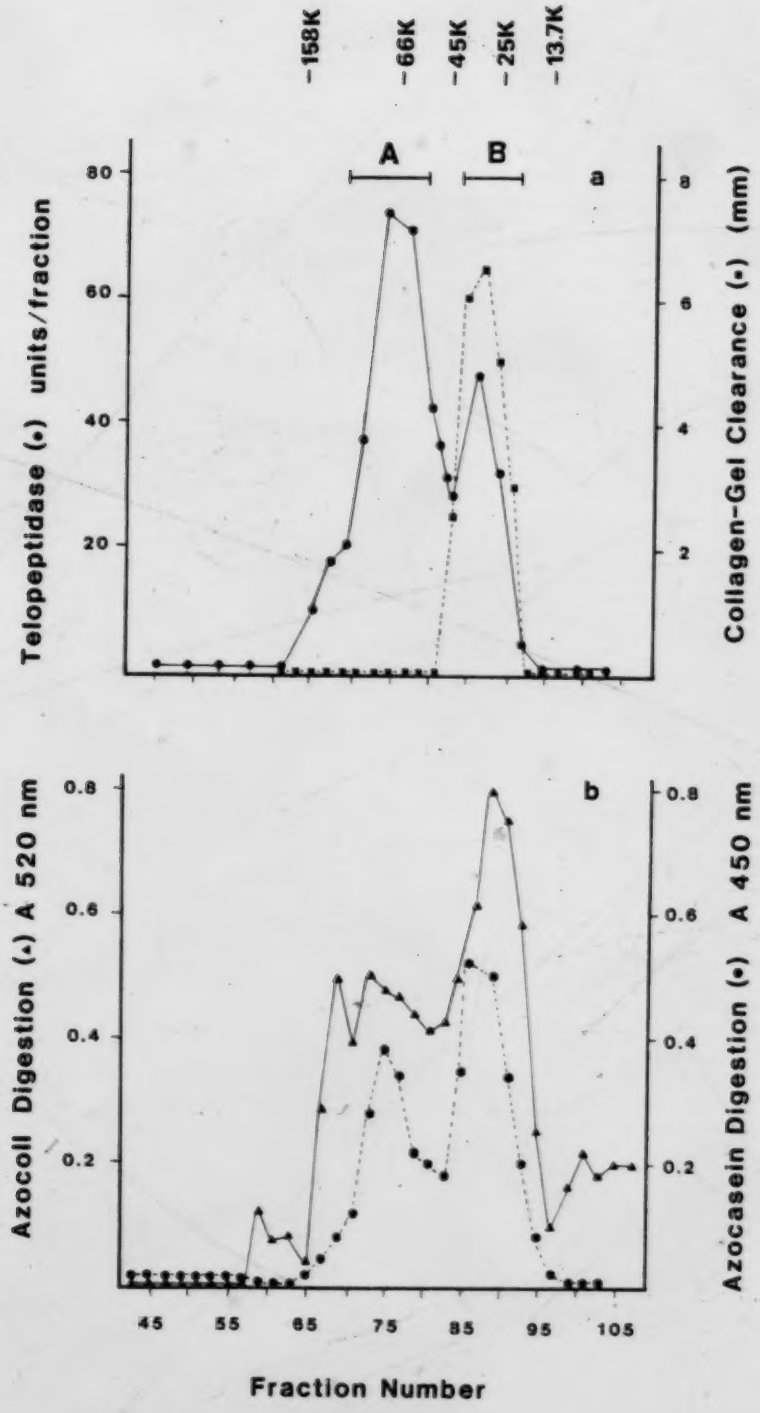
Figure 24. Elution Profiles of Proteolytic Activities of Conditioned Medium on Agarose A0.5M; Preparative Chromatography.

Concentrated (40 fold), activated (pHMB) conditioned medium (15ml), was applied to a column (2.5 x 115cm) packed with agarose A0.5M equilibrated in 0.05M-Tris/HCl pH 7.4, containing 1.0M-NaCl, 0.005M-$CaCl_2$, 0.05% Brij, and 0.03% toluene The column was eluted in this buffer at 15ml per hour. Fractions (5.0ml) were collected, and aliquots taken for the analysis of various proteolytic activities. All incubations for the determination of proteolytic activity were performed for a 20-24 hour period.

The protein standards are as described in Figure 23. While not shown on chromatogram, the ^3H_2O was eluted at fraction number 115 (peak).

A and B refers to the regions of the chromatogram, that were pooled for further investigation and, as described in the text, are referred to as:
Fraction A - which contains the telopeptidase activity with an apparent Mr of 70,000; and Fraction B - which contains the telopeptidase activity of an apparent Mr of 35,000.

a) The elution profiles of telopeptidase and collagenase activities.
b) The elution profiles of Azocoll degrading and azocasein degrading activities.


158K
66K
45K
25K
13.7K
A
B
a
Telopeptidase (•) units/fraction
Collagen-Gel Clearance (•) (mm)
b
Azocoll Digestion (•) A 520 nm
Azocasein Digestion (•) A 450 nm
Fraction Number

Fraction B was pooled, dialyzed against buffer containing no sodium chloride, and then chromatographed on DEAE-Sepharose 4B. The collagenase and telopeptidase activities were not retarded. These fractions were pooled, the conductivity was measured, and then the sample was chromatographed on a column packed with heparin-agarose (results not shown). The two proteolytic activities were retarded on the heparin-agarose, however they both were eluted with the buffer containing 0.4M-NaCl and thus not separable by this technique (this method is as described by Cawston and Tyler (1979)). It was subsequently demonstrated that purified collagenase kindly supplied by Dr. T. Cawston, contained telopeptidase activity. In fact the ratio of collagenase to telopeptidase activity in this preparation was similar to that observed in Fraction B (Table 12).

Further attempts were made to fractionate the proteinases by collagen-affinity chromatography. Fraction B was pooled, concentrated and dialyzed against running buffer containing no sodium chloride, and then applied first to a column (1.5 x 7cm) packed with DEAE-Sepharose 6B, as above, in order to remove any strongly anionic molecules that may compete with the proteinases for binding sites on collagen. The non-retarded fractions containing proteolytic activity were then applied to a column packed with the immobilized collagen. Recoveries were quite low, and it did not appear that any of the proteolytic activities was retarded. No further attempts were made to purify the collagenase.

Table 12. Ratio of Collagenase and Telopeptidase Activities in Various Fractions.

FRACTION	COLLAGENASE[b] units/ml	TELOPEPTIDASE[b] units/ml	RATIO
medium (act.)[a]	24±5	1.9±0.3	13/1
60% $(NH_4)_2SO_4$ pp't.	200±15	14.3±0.9	14/1
Fraction B	200±26	3.6±0.2	56/1
Dr. Cawston's collagenase prep.	1450±160	30.0±3.2	48/1

act. - activated; pp't. - precipitate;
prep. - preparation;
a - conditioned medium, non-concentrated, was dialyzed against assay buffer, and activated by trypsin-agarose.
b - values are of duplicate determinations with ranges.

Analysis of the Telopeptidase Activity in Fraction A

It was initially thought that the telopeptidase of apparent Mr 70,000, could be due to dimerization of the enzyme of apparent Mr 35,000, or due to some other type of aggregation. Initially the chromatography of the concentrated preparation was performed in chromatography buffer containing 1.0M-NaCl. To determine whether this high salt concentration had caused aggregation, an aliquot of Fraction A was rechromatographed and eluted in buffer containing 0.2M-NaCl (results not shown). There was no difference in the elution position of the telopeptidase activity. In addition, an aliquot of Fraction A was treated with soluble trypsin to determine whether the pHMB-activated telopeptidase could be further dissociated by enzymic means. It was observed that there was no significant difference in total telopeptidase activity after treatment with trypsin, either for 10 or 20 minutes, when compared to the zero-time control, as well as no difference in the telopeptidase elution profile (Fig 25). In subsequent experiments, the concentrated conditioned medium was activated by trypsin-agarose, and chromatographed on agarose A0.5M with chromatography buffer containing 0.2M-NaCl. The proteolytic activity elution profiles of this gel filtration run were similar to that obtained with pHMB-activated medium (see Fig 24a).

Fraction A was pooled, concentrated and rechromatographed. The elution profile of telopeptidase activity was

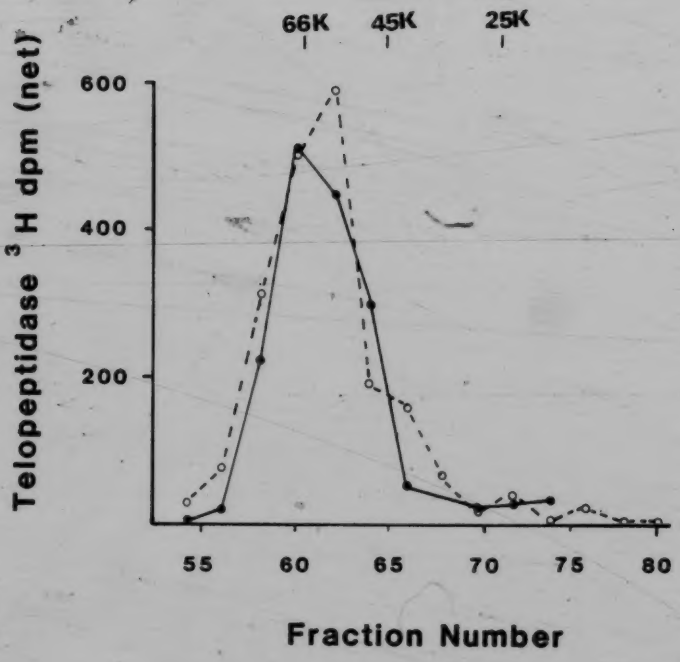


Figure 25. Elution Profiles of Telopeptidase Activity of Trypsin-Treated Fraction A.

An aliquot (1.0ml) of Fraction A (see Figure 24) was treated with trypsin in solution (10µg) for 10 minutes at 22°C. After addition of soybean trypsin inhibitor (50µg), the solution was chromatographed on a column (1 x 115cm) packed with agarose A0.5M and eluted in chromatography buffer containing 0.2M-NaCl (see Figure 23). Fractions (1.04ml) were collected and analyzed for telopeptidase activity.

(●) 0-time control; premixed soybean trypsin inhibitor and trypsin added to Fraction A.
(○) trypsin treated Fraction A.

66K - albumin; 45K - ovalbumin;
25K - chymotrypsinogen.

similar to that shown in Fig 25. Fraction A was incubated with acetic acid soluble collagen and the products electrophoresed on 5% polyacrylamide gels. These profiles (not shown) indicated the presence of collagenase. Judging by the conversion of α_1 to α,3/4 (see Fig 22), there was approximately 17.5 units of collagenase in the incubation mixture, which was known to contain 6 units of telopeptidase activity (3 to 1 ratio).

The presence of collagenase in twice-chromatographed Fraction A was unexpected since there was no clearance of the collagen (by the capillary-gel method) in the 48 hours of incubation. In a fresh preparation of conditioned medium which was processed by the standard techniques including treatment with trypsin-agarose, collagenase activity was also evident in Fraction A, but only after a long incubation period (Fig 26, 27). It can be deduced from the results shown in Fig 27 that collagenase must be present in Fraction A initially in an inactive form. Using the rate of digestion from 56 hours onwards, the amount of collagenase in Fraction A, was shown to be substantial, approximately 30% of the activity found in Fraction B.

From the results described above it was assumed that the collagenase was not activated completely although both pHMB and insoluble trypsin had been used. An aliquot of Fraction A was concentrated, treated with soluble trypsin, and the activities of collagenase and telopeptidase were determined (Table 13). As demonstrated there was a

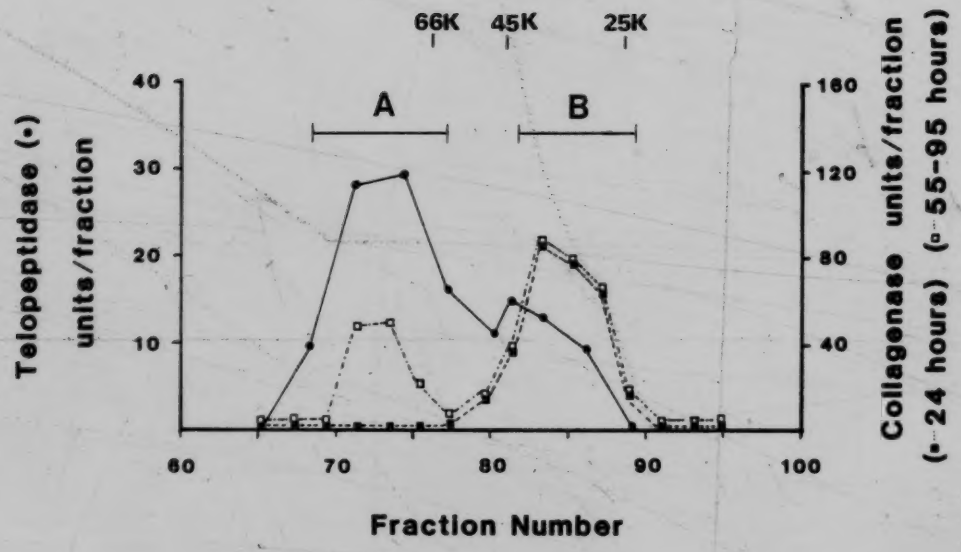


Figure 26. Elution Profile of Proteolytic Activity of Conditioned Medium.

Concentrated (25 fold), activated (trypsin-agarose) conditioned medium (12ml) was chromatographed on a column (2.5 x 115cm) packed with agarose A0.5M, in chromatography buffer containing 0.2M-NaCl. Fractions (5.0ml) were collected and aliquots analyzed for telopeptidase activity and collagenase activity. The latter activity was estimated using reconstituted collagen in capillary tubes, at 24 hours and 95 hours of incubation as noted on figure. Note that there was no evidence of collagenase activity in Fraction A in the first 24 hours of incubation (see Figure 27).

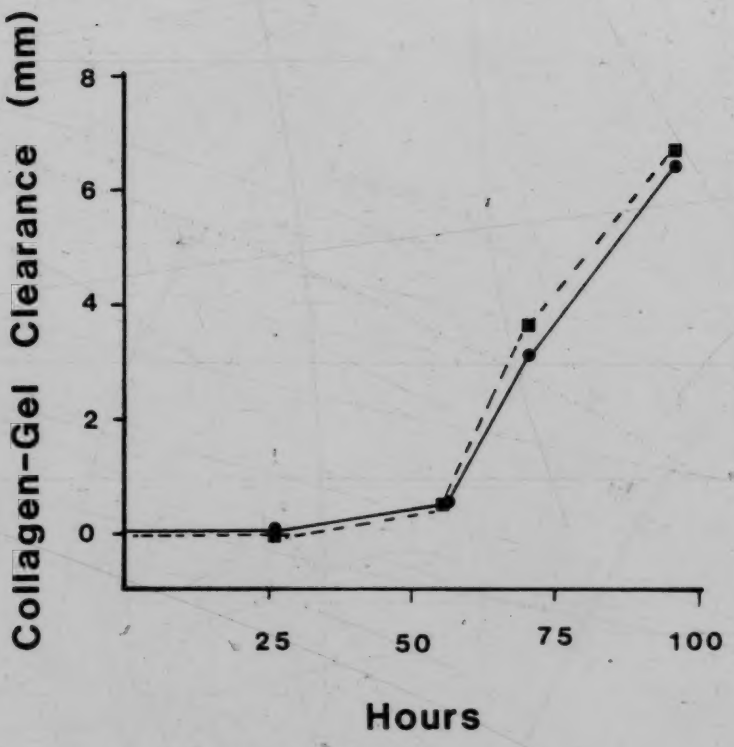


Figure 27. Clearance of Reconstituted Collagen by a 'Collagenase' of Fraction A; Demonstration of Latency.

Aliquots from fractions of Fraction A (see Fig 26) were incubated with collagen gels in capillary tubes for 95 hours.
(●) fraction number 72.
(■) fraction number 74.
Note the lag period of up to 50 hours before clearance of collagen gels was evident.
Values are results of single determinations.

Table 13. Effect of Soluble Trypsin on the Proteolytic Activities in Fraction A.

An aliquot (10ml) of Fraction A was concentrated (5 fold) and treated with trypsin (10µg) in solution for 10 minutes at 23°C. Aliquots of the pre- and post-activation enzyme preparations were compared in the ability to release low molecular weight labelled peptides, and the ability to solubilize collagen gels in capillary tubes in a 46.5 hour incubation time.

ACTIVITIES	PRE-ACTIVATION total units	POST-ACTIVATION total units	CHANGE (%)
telopeptidase[b]	38.6 ± 1.7	40.8 ± 0.2	+6[a]
collagenase[c]	146 ± 37	214 ± 29	+46
collaganase telopeptidase	3.8	5.2	+37

a - this increase is not significant; within experimental error.
b - values are the means of duplicate determinations with ranges.
c - values are the means of determinations in triplicate with ranges.

significant amount of collagenase activity in the untreated fraction, which was increased by approximately 45% after exposure to trypsin. There was no difference in telopeptidase activity. It was thought that the latent collagenase activity was being slowly activated upon standing at 4°C, and more efficiently activated when exposed to the acid soluble collagen substrate at 22°C. Addition of trypsin likely accelerated this activation.

Fraction A was treated with soluble trypsin and then re-chromatographed (Fig 28). Active collagenase was demonstrable in a 24 hour incubation on the collagen gels, in those fractions that corresponded to an apparent Mr of 35,000. However collagenase activity was still evident in Fraction A, but only after several days of incubation. Fraction A was pooled, concentrated and treated once again with soluble trypsin, and rechromatographed. The elution profiles of the proteinase activities were similar to that shown in Fig 28, with the presence of active collagenase in those fractions of an apparent Mr of 35,000 (though at a lower concentration), and latent collagenase activity in Fraction A. After 7 days of incubation with the collagen gels, clearance became evident. Thus it would appear that the collagenase contaminating Fraction A was being progressively reduced by the repeated gel filtration treatments. However it was also evident that trypsin was not totally effective in activating all the latent collagenase.

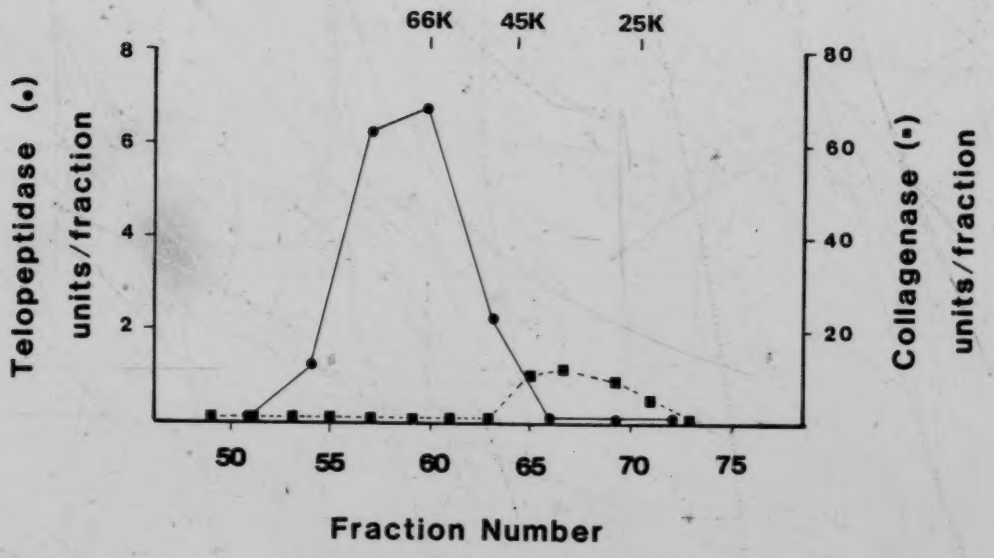


Figure 28. Chromatography of Fraction A; Elution Profiles of Proteolytic Activities.

A sample (10ml) of Fraction A was concentrated (5 fold) and treated with trypsin in solution (20µg) for 15 minutes, followed by the addition of soybean trypsin inhibitor (100µg). The sample was chromatographed on a column (1 x 115cm) packed with agarose A0.5M equilibrated in chromatography buffer. Aliquots were analyzed for telopeptidase activity and for collagenase activity. Note that there was no evident collagenase activity in Fraction A, in the first 48 hours of incubation.

G. Incubation of Fraction A with the Collagen Substrates

Acid Soluble Collagen Digestions

Acid soluble collagen was incubated with a preparation of Fraction A which had been treated twice with soluble trypsin and soybean trypsin inhibitor, with intervening gel filtrations to remove any active collagenase. Electrophoresis of the collagen on 5% polyacrylamide gels demonstrated the presence of a minor level of collagenase activity (Fig 29c,d). In addition, the relative proportion of the β components and α chains do not appear to be different between the digested collagen (with Fraction A) and the no-enzyme control, as opposed to the effects noted with pepsin digested collagen (Fig 29b). An EDTA-inhibited conditioned medium control (not shown) had a similar electrophoretic profile to that of the no-enzyme control.

The effects of Fraction A on the collagen, were further characterized by treatment of the products with cyanogen bromide and electrophoresis on 12% polyacrylamide gels (Fig 30). The cyanogen bromide peptides of pepsin digested collagen, in which virtually all the α_1CB6 was shortened, were used as the positive control. In this way, the amount of shortening caused by the partially purified proteinase preparation could be estimated (Table 14). However, since there was an incomplete separation of the α_1CB6 peptides from each other, the level of shortening that was calculated is only an approximation. Also included in Table 14, is the

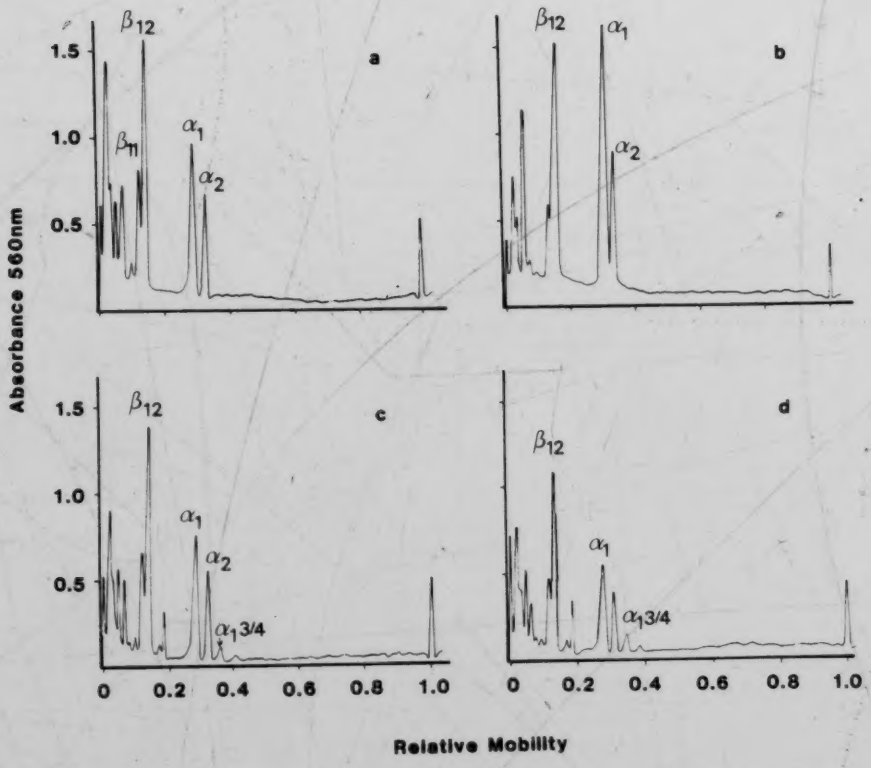


Figure 29. Gel Electrophoresis of Products of Incubation of Acid Soluble Collagen and Fraction A.

Acid soluble collagen (400µg) was incubated with aliquots of Fraction A in assay buffer (final volume 4.0ml) for 29 hours at 22°C. The reactions were terminated by the addition of EDTA (0.03M), samples dialyzed, lyophilized, and a portion electrophoresed on 5% polyacrylamide SDS gels.

a) no-enzyme control.
b) acid soluble collagen digested by pepsin (control)
c) acid soluble collagen incubated with 6.6 units of telopeptidase activity.
d) acid soluble collagen incubated with 13.2 units of telopeptidase activity.

Note there was still a low level of collagenase activity in Fraction A.

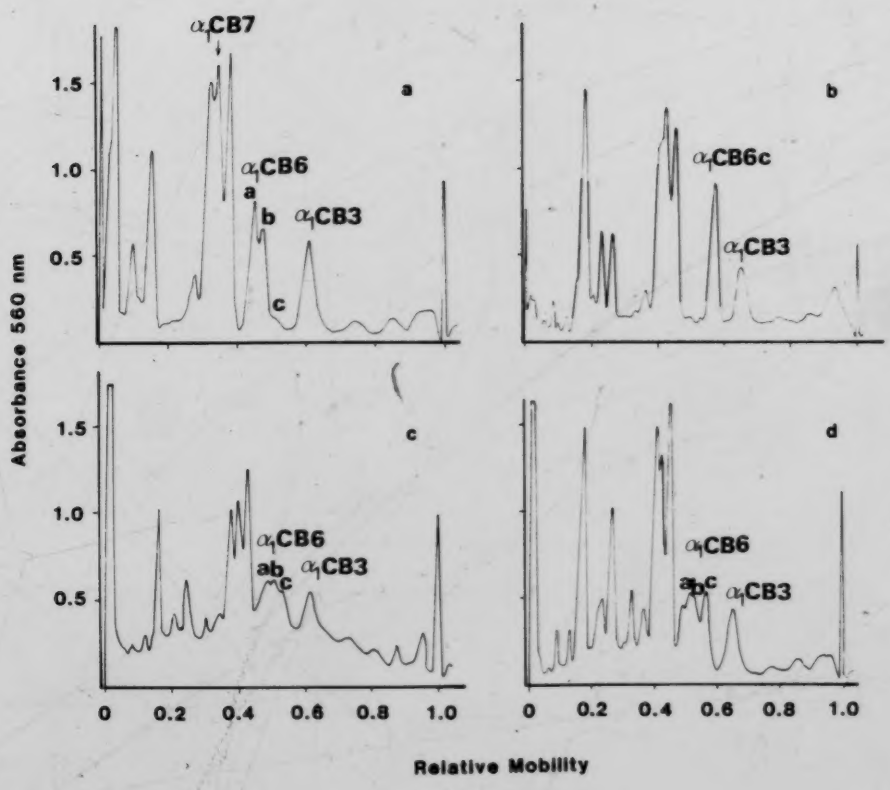


Figure 30. Gel Electrophoresis of Cyanogen Bromide Digested-Products of Incubation of Acid Soluble Collagen and Fraction A.

Acid soluble collagen was incubated with Fraction A as described in Figure 29. The lyophilized samples were treated with cyanogen bromide and the peptides electrophoresed on 12% polyacrylamide SDS gels.

a) no-enzyme control.
b) collagen digested by pepsin.
c) collagen incubated with 6.6 units of telopeptidase activity.
d) collagen incubated with 13.2 units of telopeptidase activity.

Note that pepsin converted all of the α_1CB6 peptides into the shortened form - α_1CB6c.

In addition, note the presence of a significant level of α_1CB6c in the test incubation.

Table 14. Effects of Proteolytic Activity of Fraction A on the Collagen Substrate.

Acid soluble collagen was incubated with Fraction A and the products of the digestion analyzed as described in Figures 29 and 30. Collagenase activity was determined by the conversion of α_1 to $\alpha_1 3/4$ as described in Methods (collagenase assay). The percent shortening was calculated as described below. The values are of duplicate determinations with ranges where indicated.

	COLLAGENASE		TELOPEPTIDASE [c]
FRACTION A (volume)[a]	conversion(%)	units [b]	α_1CB6c/α_1CB6 (%)
1.5ml	11.9 ± 1.1	1.84 ± 0.18	36
3.0ml	17.3 ± 2.1	2.74 ± 0.33	53 ± 2

a - approximately 4.4 units of telopeptidase activity/ml based on the ^{3}H-collagen assay
b - numbers based on effect; expressed as µg collagen digested per hour at 22°C.
c - the percent shortening was calculated as follows:

$$[\alpha_1 CB6c(pepsin) - \alpha_1 CB6c(control)] / \alpha_1 CB3 = Y$$

$$[\alpha_1 CB6c(test) - \alpha_1 CB6c(control)] / \alpha_1 CB3 = X$$

$$(X / Y) \times 100\% = \text{relative level of shortening (\%).}$$

estimated level of conversion of the α_1 chains to $\alpha_1$3/4 peptides (a measure of collagenase activity). As shown approximately 36 and 53% of the α_1CB6 was converted to the shortest form by the action of increasing amounts of the Fraction A, while the level of collagenase activity was only one third of the telopeptidase activity at each concentration. In terms of units of activity when expressed as μg of collagen digested per hour, by both activities, the relative ratios are also similar to the above. Approximately 1μg of collagen was shortened at the C-terminus for every 0.3μg that was digested by collagenase.

^{3}H-Collagen Incubation with Fraction A

For additional proof that increased levels of the band identified as the α_1CB6c peptide was a direct result of removal of the carboxy-terminal extra-helical region (or portion thereof), Fraction A was incubated with the ^{3}H-collagen. However, when the proteinase solution was first dialyzed against ammonium propionate buffer according to the previously established protocol, the majority of the telopeptidase activity was lost. Desalting the proteinase preparation on Sephadex G25 in this buffer also resulted in unacceptably high losses of activity.

A different method was attempted. An aliquot of Fraction A (the fraction used was one that had been chromatographed twice on agarose A0.5M without treatment with trypsin) was incubated with the ^{3}H-collagen in the

standard assay buffer. The digestion appeared to have gone to completion by virtue of the relative amount of radioactivity eluting in the Vt fraction when an aliquot (5% of the incubation mixture) was chromatographed on Bio-Gel P4. The incubation mixture was then dialyzed overnight against 50 volumes of 0.02N-acetic acid. The level of radioactivity recovered in the diffusate was equivalent to 95% or greater of the expected level based on the Bio-Gel P4 chromatography. The diffusate was lyophilized and an aliquot run on the amino acid analyzer after hydrolysis (Fig 31c). Only a small percentage (5.5%) of the initial diffusate was applied to the analyzer due to the potential overloading of the resin with salt. The retentate, or large molecular weight fraction was dialyzed an additional 24 hours against 100 volumes of 0.02N-acetic acid, and analyzed as above (Fig 31b). As can be observed from the ^{3}H-amino acid elution profiles, there was a definite decrease in ^{3}H-hydroxynorleucine and ^{3}H-chlornorleucine in the high molecular weight fraction after digestion with Fraction A, and the appearance of the above in the fraction that was diffusible (dialysis tubing of 6-8,000 molecular weight cutoff was used). In Table 15, the relative levels of ^{3}H-hydroxynorleucine and ^{3}H-chloronorleucine are shown. Approximately 62% of the specific radioactivity (in the form of hydroxynorleucine and chlornorleucine) was removed. Amino acid analysis of the low molecular weight fraction did not demonstrate the presence of hydroxyproline (with a known sensitivity limit of 2.0% of

Figure 31. ^{3}H-Amino Acid Elution Profiles of the Fractionated Products of Incubation of ^{3}H-Collagen and Fraction A.

Fraction A (11 units of telopeptidase activity) was incubated with ^{3}H-collagen (200µg) in assay buffer (final volume 2.4ml) for 29 hours at 22°C. After dialysis against 50 volumes of 0.02N-acetic acid for 24 hours, the diffusate was lyophilized and an aliquot (20%) hydrolyzed in 5.7N-HCl. The retentate was dialyzed a further 24 hours against 100 volumes of acetic acid, lyophilized, and an aliquot (50µg) hydrolyzed in HCl and analyzed along with the diffusate on the amino acid analyzer. Fractions (0.5ml) were collected and the radioactivity measured.

a) no-enzyme control.
b) retentate or large molecular weight fraction (equivalent to the Vo fraction). A sample equivalent to 25% of the initial starting substrate was applied to the analyzer.
c) diffusate or low molecular weight fraction (equivalent to the Vt fraction). A sample equivalent to 5.5% of the initial substrate was applied to the analyzer.

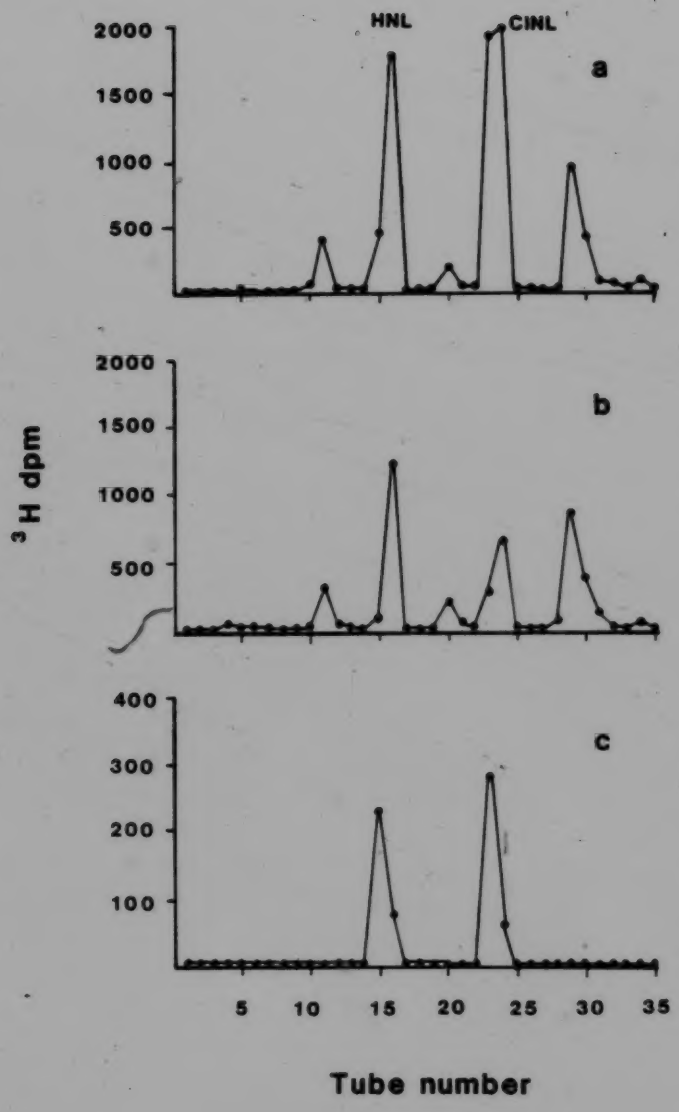

HNL
CINL
a
b
c
2000
1500
1000
500
400
300
200
100
^{3}H dpm
5
10
15
20
25
30
35
Tube number

Table 15. Effect of Fraction A on ^{3}H-Collagen; Release of Low Molecular Weight Peptides Containing ^{3}H-Hydroxynorleucine.

Fraction A was incubated with ^{3}H-collagen as described in Figure 30. The amount of radioactivity associated with the cross-link precursor - hydroxynorleucine - is expressed as a function of hydroxyproline concentration. Values are the results of single determinations.

TEST	HNL + ClNL (dpm)	HYP(nmole)	$\frac{dpm}{nmole}$	HNL RELEASED (%)
no-enzyme(Vo)	6214	38.50	161	0
digest (Vo) [a]	2260	36.75	61.4	62
digest (Vt) [b]	2832	0	-	-

a - the amount of sample analyzed from the Vo fraction was representative of 25% of the starting material.

b - the amount of sample analyzed from the Vt fraction was representative of only 5.5% of the starting material, however the level of radioactivity shown are the adjusted values assuming 25% of the material was analyzed.

the starting material under the conditions of this experiment). It has been shown that at least two-thirds of the ^{3}H-hydroxynorleucine in the reduced collagen is situated at the C-terminal extra-helical region (see Table 4; cathepsin D digested ^{3}H-collagen). As well, incubations of acid soluble collagen with Fraction A did not reveal an activity that was directed towards the N-terminal extra-helical region (see Fig 29). Thus based on the above evidence, as well as on the calculated molecular weight of the α_1CB6c (17,200 daltons; Fig 30), the activity present in Fraction A was acting at the carboxy-terminal extra-helical region.

The remainder of the large molecular weight (Vo) fraction (150μg) as well as a no-enzyme control of ^{3}H-collagen, were treated with cyanogen bromide and the products electrophoresed on 12% polyacrylamide gels (Fig 32). The profile of the control demonstrates the presence of the three forms of α_1CB6 as described previously. The profile of the collagen that was incubated with Fraction A was more complex. The shortest form of α_1CB6 (α_1CB6c), was seen in the concentration that results from 90-100% shortening (this value was obtained upon comparison of the values demonstrable with pepsin digested collagen - see above). However a prominent band was observed with an apparent Mr of 18,400 which is similar in relative mobility to that of the α_1CB6b. The possible origin of this is discussed later. Some collagenase activity was evident by

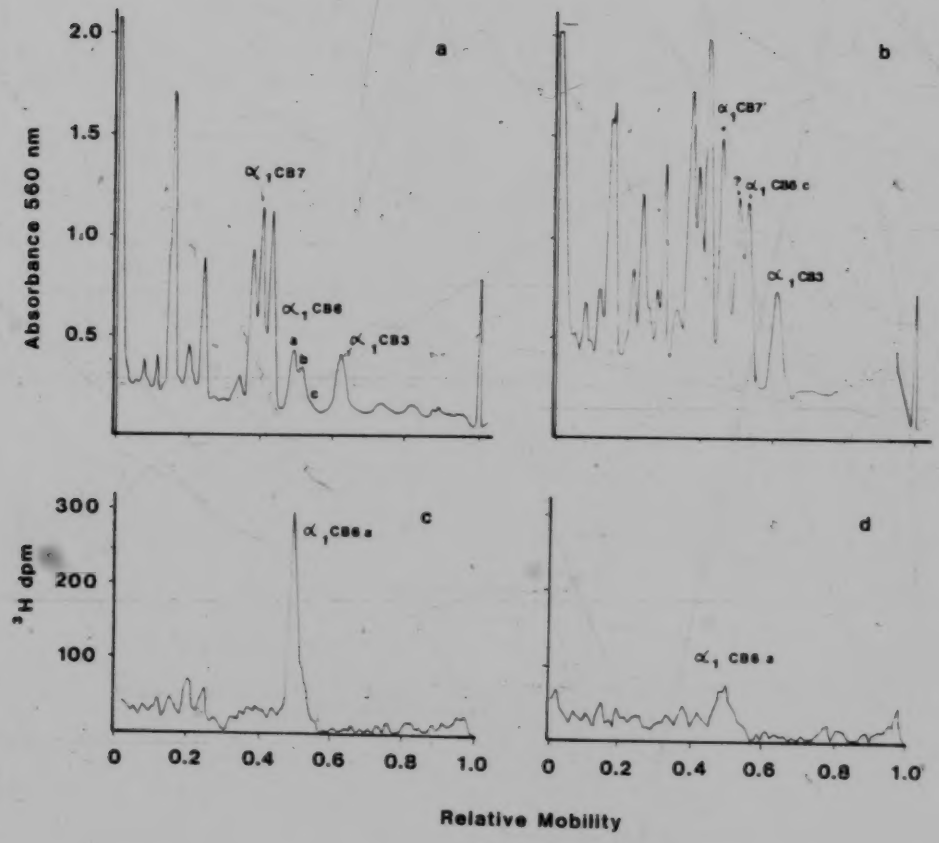


Figure 32. Gel Electrophoresis of Cyanogen Bromide Digested-Products of the Incubation of ^{3}H-Collagen and Fraction A.

Fraction A was incubated with ^{3}H-collagen as described in Figure 31. The retentate (150µg), and a no-enzyme control were digested with cyanogen bromide, and the products electrophoresed on 12% polyacrylamide SDS gels. After scanning, the gels were frozen, sliced into 1mm section (as described in Methods), peptides solubilized and radioactivity measured.

a,b - Absorbance at 560nm.
c,d - ^{3}H-dpm in each gel slice.

a,c - no enzyme control.
b,d - collagen incubated with Fraction A.

Note the decrease in the level of radioactivity associated with the α_1CB6a peptide after digestion with Fraction A.

virtue of the decreased amount of α_1CB7 (contains the collagenase cleavage site; see Fig 2). To demonstrate the loss of the specific radioactivity associated with α_1CB6a, the radioactivity associated with the peptide bands were measured (Fig 32c,d). Only a small fraction (10-20%) of the initial radioactivity associated with the α_1CB6a peptide was left after digestion with Fraction A.

Acid Insoluble Collagen Incubations with Fraction A

Acetic acid insoluble collagen was incubated with Fraction A for 47 hours at 22°C. This enzyme preparation was the same as described for the non-radioactive acid soluble collagen incubations. As shown in Table 16, a significant proportion of the collagen was released, however a linear relationship between the amount of enzyme added and degree of solubilization was not seen. Both the neutral salt and acetic acid extracts were examined on 5% polyacrylamide gels (Fig 33) and, after cyanogen bromide digestion, on 12% polyacrylamide gels (Fig 34). It was apparent that the conversion of α_1 to $\alpha_1$3/4 peptides (of the extracted collagen) could not have been more than 10%, and was very likely less than this. However analysis of the cyanogen bromide peptides of the solubilized collagen demonstrated that there was a significant amount of α_1CB6c peptide (Table 17).

Table 16. Solubilization of Collagen; Incubations of Acetic Acid Insoluble Collagen and Fraction A.

Aliquots of Fraction A were incubated with acid insoluble collagen as described in Figure 33. The amount of collagen solubilized is shown as a percentage (%) of the starting dry weight of collagen. Values are the results of single determinations.

TELOPEPTIDASE (units)	NEUTRAL SALT EXTRACTS(%)	ACETIC ACID EXTRACT(%)	TOTAL	TOTAL LESS CONTROL
no-enzyme	0.29	1.78	2.07	0
13.2 + 0.035M-EDTA	1.19	2.26	3.45	1.38
6.6	2.95	4.00	6.95	4.88
19.8	4.44	3.91	8.35	6.28

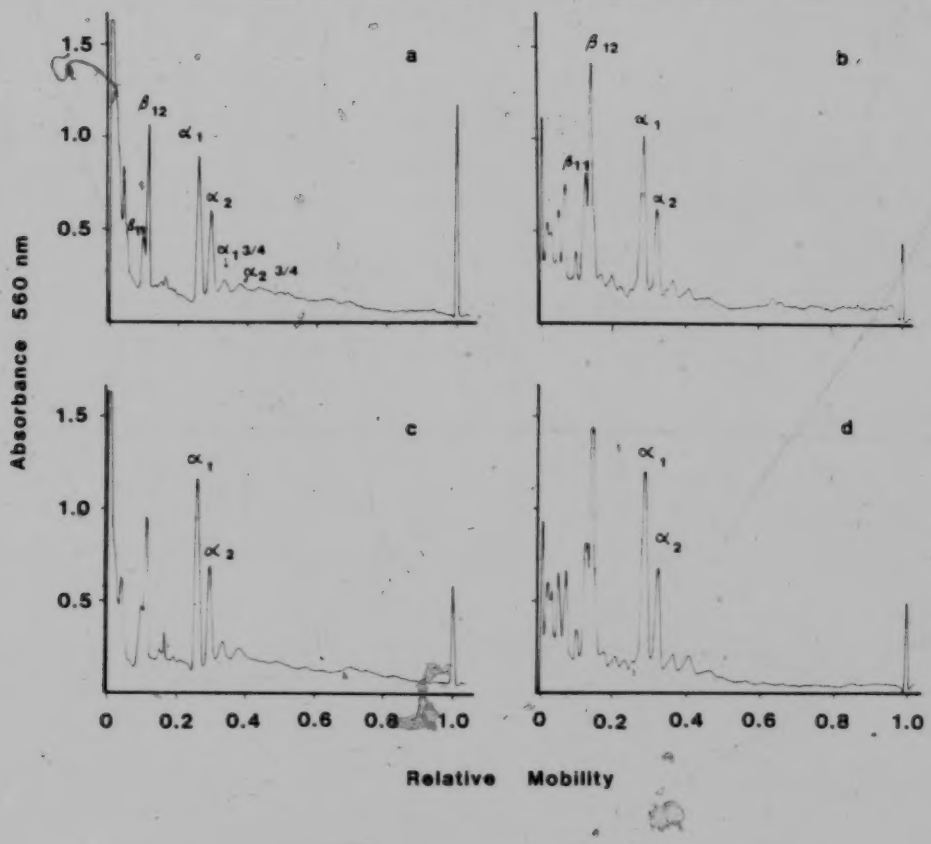


Figure 33. Gel Electrophoresis of the Products of Incubation of Acid Insoluble Collagen and Fraction A.

Aliquots of Fraction A (see Table 16) were incubated with acid insoluble collagen (27mg) in assay buffer (final volume 4.0ml) for 47 hours at 22°C. The reaction was terminated by the addition of EDTA (0.03M). The neutral salt and water extracts, which were pooled, and the acetic acid extract, were processed as described in Methods, and products electrophoresed on 5% polyacrylamide SDS gels.

a) neutral salt extract of incubation containing 6.6 units of telopeptidase activity.
b) acetic acid extract of collagen residue of (a)
c) neutral salt extract of incubation containing 19.8 units of telopeptidase activity.
d) acetic acid extract of collagen residue of (c).

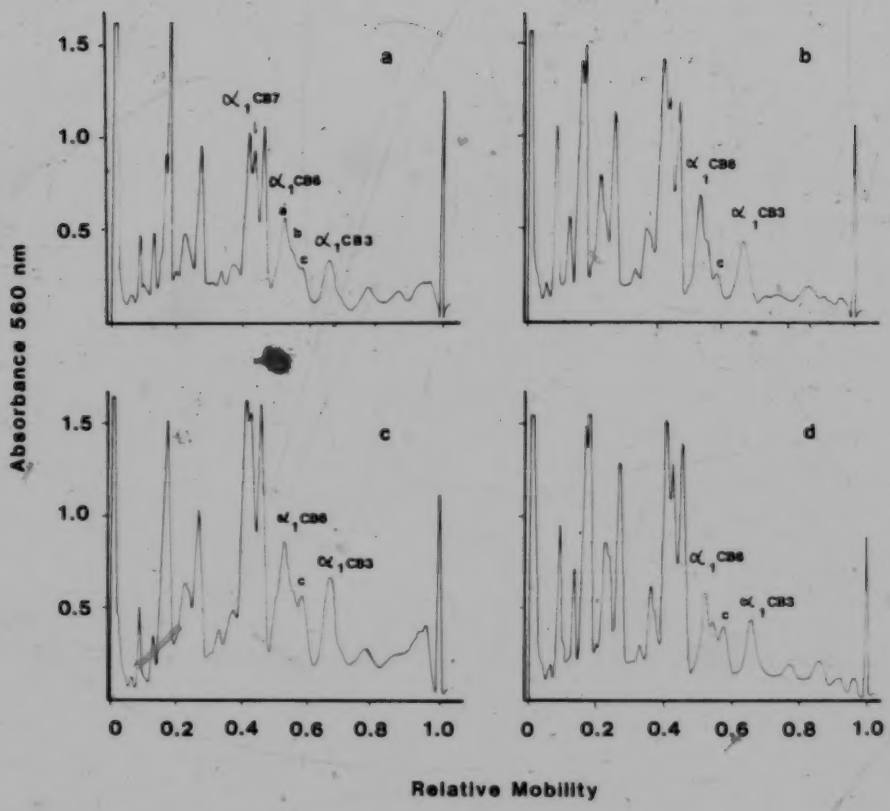


Figure 34. Gel Electrophoresis of Cyanogen Bromide Digested-Products of the Incubation of Acid Insoluble Collagen and Fraction A.

Acetic acid insoluble collagen was incubated with Fraction A, and the products dialyzed and lyophilized (see Figure 33). Samples of each extract were digested with cyanogen bromide and the products electrophoresed on 12% polyacrylamide SDS gels.

a) neutral salt extract of incubation containing 6.6 units of telopeptidase activity.
b) acetic acid extract of collagen residue of (a)
c) neutral salt extract of incubation containing 19.8 units of telopeptidase activity.
d) acetic acid extract of collagen residue of (c).

Table 17. Incubation of Fraction A with Acid Insoluble Collagen; Quantitation of α_1CB6c.

Acid Insoluble collagen was incubated with Fraction A (see Figure 33, Table 16) and the products digested by cyanogen bromide (see Figure 34). The degree of 'shortening' of α_1CB6 was quantitated as described in Table 14. Values are the results of duplicate determinations with ranges.

TELOPEPTIDASE (units)	EXTRACT FRACTION	SHORTENING [a] (%)
6.6	neutral salt	18.7±6.0
6.6	acetic acid	9.4±1.3
19.8	neutral salt	35.7±0.3
19.8	acetic acid	30.7±0.7

a - after correction for no-enzyme control

H. Incubation of Fraction B with the Collagen Substrates

The various collagen preparations were incubated with Fraction B. The ratio of collagenase to telopeptidase activity was approximately 690/1, based on the conversion of α_1 to $\alpha_1$3/4 peptides on gel electrophoresis and the telopeptidase assay respectively. Thus to determine the effects of telopeptidase activity on the collagen substrates, a huge excess of collagenase activity was present in the incubations.

Incubations with acetic acid insoluble collagen resulted in the solubilization of a significant amount of collagen (Table 18). However, again a linear relationship between the amount of added enzyme and degree of solubilization was not seen. Analysis of the neutral salt extracts demonstrated that all the collagen chains were converted to the characteristic collagenase cleavage products (Fig 35), but approximately 60% conversion was evident in the acetic acid extracts. Analysis of the cyanogen bromide digested collagen peptides from both extracts, revealed that determination of the extent of shortening of α_1CB6 was not possible (Fig 36). The presence of a large amount of a peptide with an apparent Mr of 18,400 that had the same relative mobility as α_1CB6b, partially or completely obscured the α_1CB6 peptides.

Acid soluble collagen was incubated with an aliquot of Fraction B, and the products analyzed by gel electrophoresis (Fig 37a,c). All γ components, as well as the majority of

Table 18. Solubilization of Collagen from the Incubation of Acetic Acid Insoluble Collagen and Fraction B.

Aliquots of Fraction B were incubated with acid insoluble collagen as described in Figure 35. The collagen that was extracted in the neutral salt buffer and acetic acid, was estimated by hydroxyproline analysis. The amount is expressed as a percentage of the starting dry weight of the insoluble collagen. Values are the results of single determinations.

TELOPEP-TIDASE (units)	COLLA-GENASE (units)	NEUTRAL SALT EXTRACTS (%)	ACETIC ACID EXTRACT (%)	TOTAL (%)	TOTAL Less CONTROL (%)
no-enzyme control		0.29	1.78	2.07	0
3.6	2484	1.44	6.22	7.66	5.59
7.2	4968	1.92	7.05	8.97	6.90
10.8	7452	2.34	6.98	9.32	7.25

Figure 35. Gel Electrophoresis of the Products of Incubation of Acid Insoluble Collagen and Fraction B.

Acid insoluble collagen (27mg) was incubated with Fraction B (see Table 18), in assay buffer (final volume 4.0ml) for 47 hours at 22°C. The reaction was terminated with EDTA (0.03M). The collagen was extracted in neutral salt buffer and acetic acid, and the products electrophoresed on 5% polyacrylamide SDS gels.

a) Fraction B containing 10.8 units of telopeptidase and approximately 7,500 units of collagenase, incubated in the presence of 0.03M-EDTA.
b) neutral salt extract of collagen released by the action of Fraction B (same units of activity as (a)).
c) acetic acid extract of the collagen residue of (b).

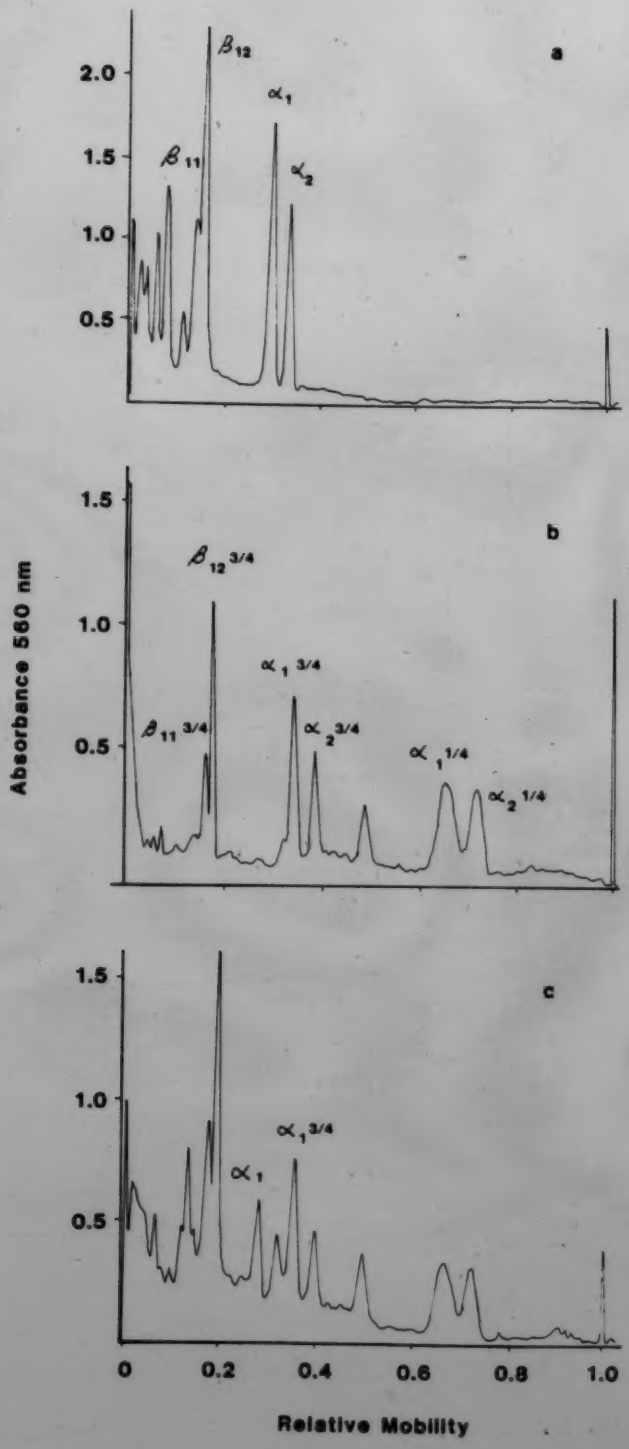

a
β_{12}
α_1
β_{11}
α_2
2.0
1.5
1.0
0.5
b
$\beta_{12}^{3/4}$
$\alpha_1^{3/4}$
$\beta_{11}^{3/4}$
$\alpha_2^{3/4}$
$\alpha_1^{1/4}$
$\alpha_2^{1/4}$
1.5
1.0
0.5
c
$\alpha_1^{3/4}$
α_1
1.5
1.0
0.5
0
0.2
0.4
0.6
0.8
1.0
Absorbance 560 nm
Relative Mobility

Figure 36. Gel Electrophoresis of Cyanogen Bromide Digested-Products of the Incubation of Acid Insoluble Collagen and Fraction B.

The products of the incubations of Fraction B and the collagen substrate (see Figure 35 for details), were digested with cyanogen bromide, and products electrophoresed on 12% polyacrylamide SDS gels.

a) acetic acid extract of the incubation containing Fraction B and 0.03M-EDTA.
b) neutral salt extract of collagen released by the action of Fraction B.
c) acetic acid extract of the collagen residue of (b).

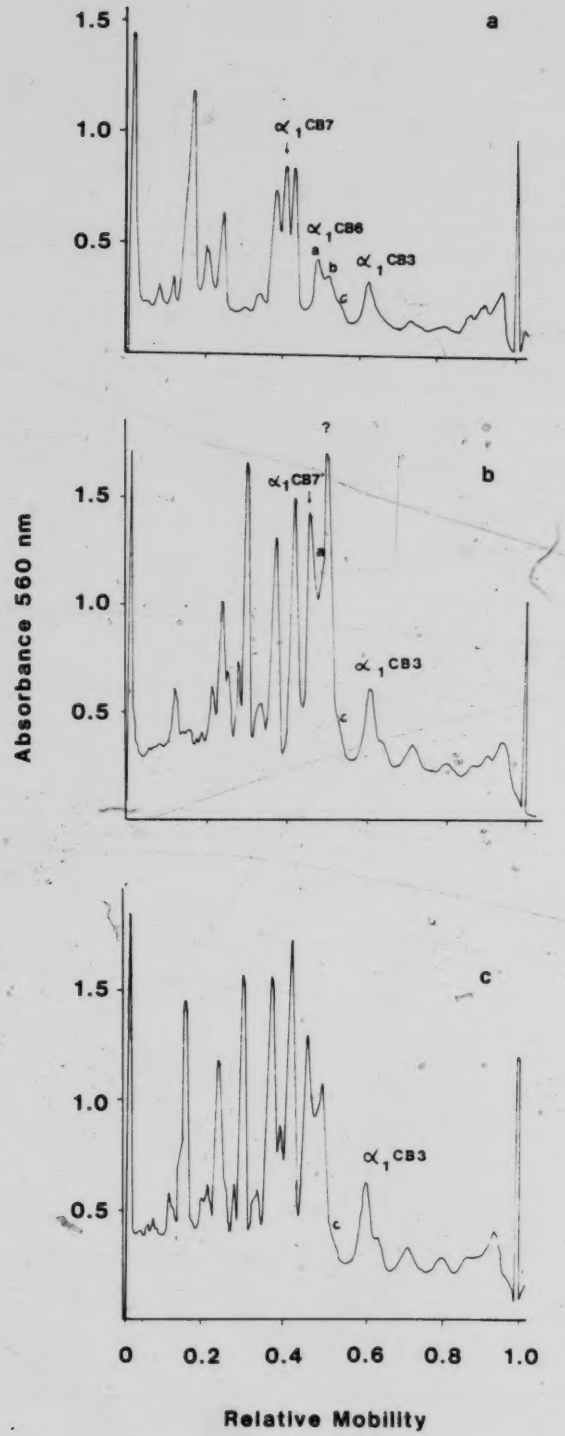
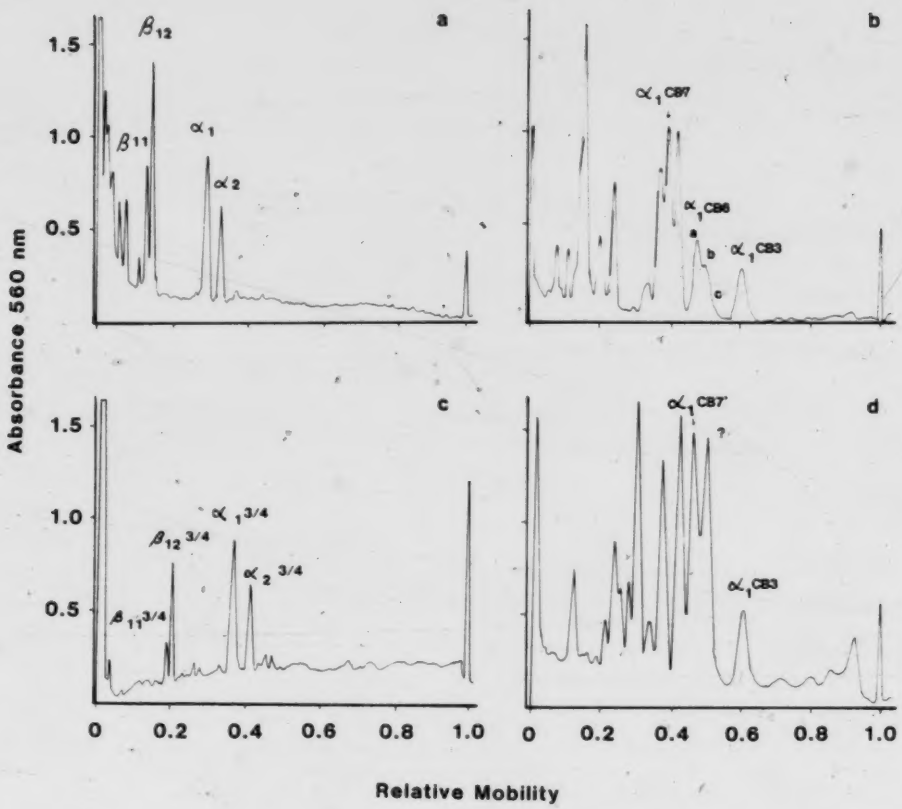

a
b
c
1.5
1.0
0.5
α_1CB7
α_1CB6
α_1CB3
a
b
c
?
Absorbance 560 nm
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility

Figure 37. Gel Electrophoresis of the Products of Incubation of Acid Soluble Collagen and Fraction B.

Acid soluble collagen (400µg) was incubated with Fraction B (1.0ml containing 3.6 units of telopeptidase activity and approximately 2500 units of collagenase), in assay buffer (final volume 4.0ml) for 29 hours at 22°C. The reaction was terminated by the addition of EDTA (0.03M), and the products either electrophoresed on 5% polyacrylamide SDS gels, or on 12% polyacrylamide SDS gels after treatment with cyanogen bromide.

a) Fraction B incubated with the collagen in the presence of 0.03M-EDTA; electrophoresed on 5% polyacrylamide SDS gels.
b) cyanogen bromide digested EDTA-inhibited control (see a); electrophoresed on 12% polyacrylamide SDS gels.
c) Fraction B incubation with the collagen; electrophoresed on 5% polyacrylamide SDS gels.
d) cyanogen bromide digested products of the incubation of Fraction B and the collagen substrate; electrophoresed on 12% polyacrylamide SDS gels.

Note the loss of essentially all of the $\alpha_1$1/4 peptides in the test incubation (c), and the absence of peptides corresponding to α_1CB6a and α_1CB6c (d).


a
b
c
d
β_{12}
β_{11}
α_1
α_2
α_1CB7
α_1CB6
α_1CB3
$\beta_{12}^{3/4}$
$\beta_{11}^{3/4}$
$\alpha_1^{3/4}$
$\alpha_2^{3/4}$
1.5
1.0
0.5
0
0.2
0.4
0.6
0.8
1.0
Absorbance 560 nm
Relative Mobility

the α1/4 peptides were apparently lost (Fig 37c). The α_1CB6a and α_1CB6c peptides of the cyanogen bromide digest of the products of incubation, were also not seen (Fig 37b). While the presence of the unknown peak (?) likely obscures the α_1CB6 peptides, if α_1CB6a and c peptides were present they should have been observed at least as shoulders on the unknown peak, as was noted in the acid insoluble collagen incubations (Fig 36b). Presumably the cause of the 'over-digestion' was the very high levels of enzyme activity in the incubations.

The proteolytic activity of Fraction B may have had an action at the N-terminal telopeptide proximal to the cross-link. The relative amount of β components and α chains (β/α) in the acid soluble collagen digestions (Fig 37c) does appear to be somewhat reduced. However, while this was not observed in the insoluble collagen digestions (Fig 35; which is not proof of the absence of an activity directed against the N-terminal telopeptide), and because of the extra digestion of the collagen that did occur, it cannot unequivocally be stated from these experiments which telopeptide region of the collagen molecule is a substrate for the proteolytic activity of Fraction B.

The radiolabelled acid soluble collagen (200μg) was incubated with Fraction B (2.0ml) for 24 hours at 22°C, in 0.1M-ammonium propionate, pH 7.2, containing 0.01M-$CaCl_2$ and 0.02% sodium azide. The high and low molecular weight fractions were separated by chromatography on Bio-Gel P4 and

analyzed after hydrolysis on the amino acid analyzer. Approximately 38% of the total ^{3}H-hydroxynorleucine was removed by the proteolytic activity, without any significant hydroxyproline associated with the low molecular weight fraction (Vt). While this is in itself a significant result, it still cannot be stated that that the telopeptidase activity of Fraction B was directed towards the carboxy-terminal extra-helical region.

I. Other Characteristics of the Telopeptidase Activities

Fraction B had been chromatographed twice on the gel filtration column. Fraction A had been chromatographed three times and treated with both insoluble and soluble trypsin and soybean trypsin inhibitor as discussed above.

Aliquots of the two pools of proteinases were adjusted with a concentrated solution of Tris/maleate of varying pH, and incubated with the ^{3}H-collagen substrate (Fig 38). As was shown, the pH optimum for telopeptidase activity in both Fractions was between 7.0-7.5.

The effects of some proteinase inhibitors on the telopeptidase activities was tested using the ^{3}H-collagen substrate (Table 19). As shown, of the inhibitors tested only EDTA had a significant effect.

The telopeptidase activity of Fraction A appeared to be relatively stable in the presence of ^{3}H-collagen for up to 48 hours (Fig 39a), which was relevant for the long term incubations with the collagen substrates. Under similar

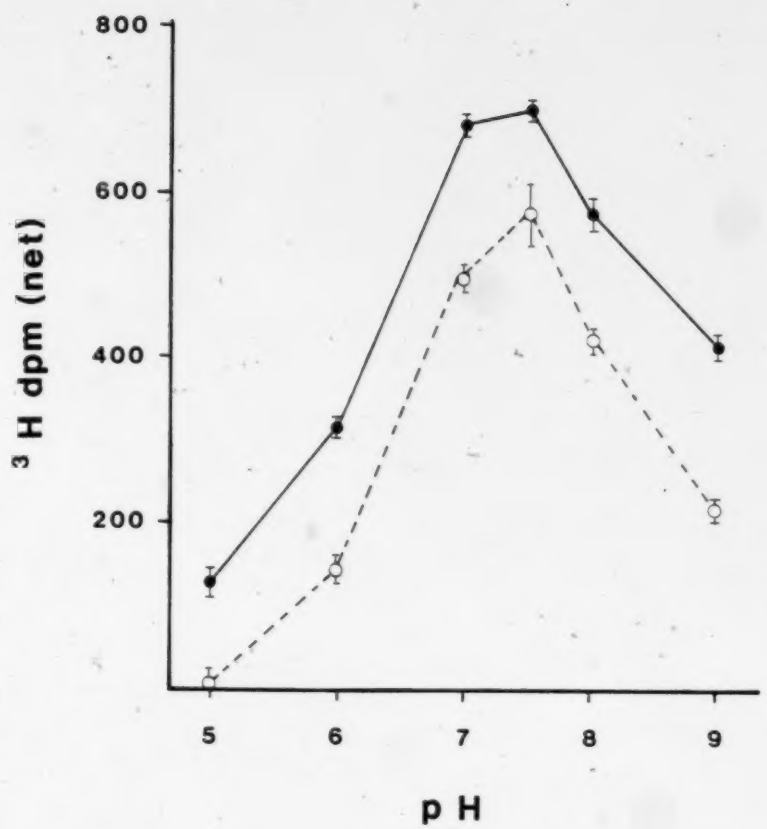


Figure 38. Telopeptidase Activity - pH Optimum.

Each of the incubation mixtures, contained the following in final concentration: 0.125M-Tris, O.125M-maleic acid, 0.133M-NaCl, 0.0033M-$CaCl_2$ and 0.014% sodium azide. In addition Brij 35 and toluene were present in the enzyme preparation mixtures at a final concentration of 0.015% and 0.01% respectively for Fraction A, and 0.02% and 0.012% respectively for Fraction B. The concentrated Tris/maleate buffer added to each incubation mixture was initially adjusted with NaOH to the pH giving the final pH of the incubation mixture shown in the figure. The incubation was carried out following the standardized protocol (Methods) for 18 hours at 22°C.
(●) - Fraction A.
(○) - Fraction B.
Values are results of duplicate determinations with ranges.

Table 19. Effects of Various Reagents on the Telopeptidase Activities of Fraction A and Fraction B.

Each of the reagents listed below were equilibrated with the ^{3}H-collagen before addition of the enzyme preparation. The mixtures were incubated for 20 hours at 22°C, in assay buffer (final volume 240µl). The results are expressed as a % of the control. The values are the means of duplicate determinations with ranges.

TEST	FRACTION A (%)	FRACTION B (%)
control	100	100
EDTA - 0.02M	5±3	10±5
NEM - 0.002M	111±1	114±15
PMSF - 0.002M	85±23	101±6

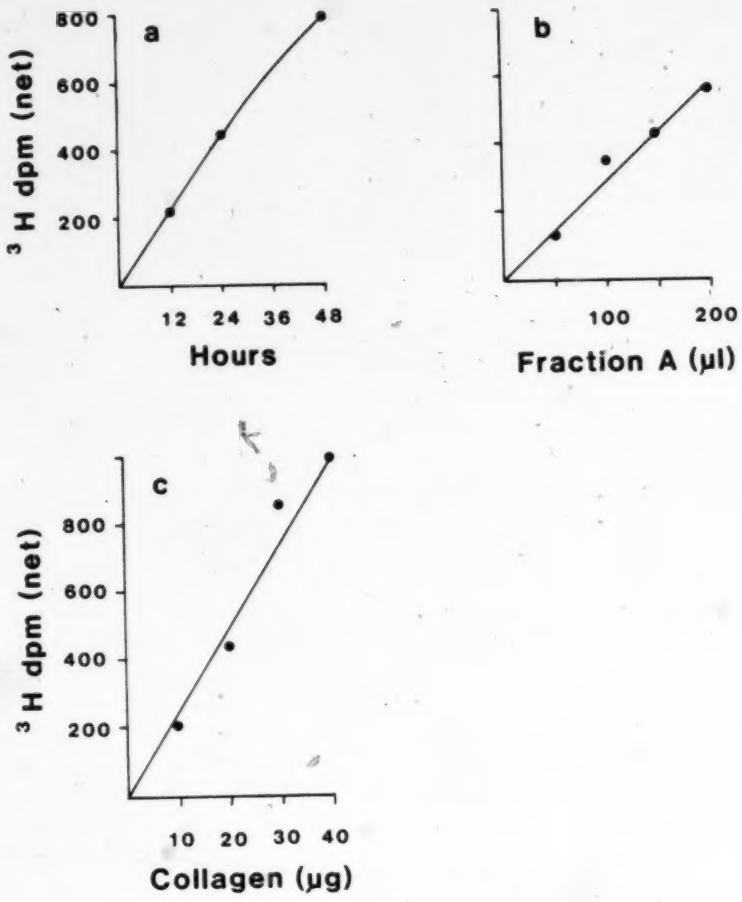


Figure 39. Other Characteristics of the Telopeptidase Activity of Fraction A.

a) Fraction A (35µl) was incubated in duplicate with the ^{3}H-collagen (20µg) at 22°C, for varying times.
b) Fraction A (varying volumes) was incubated with the ^{3}H-collagen for 24 hours at 22°C.
c) Fraction A (150µl) was incubated with varying concentrations of ^{3}H-collagen for 24 hours at 22°C.

Values are results of single determinations.

incubation conditions, the effect of varying the enzyme concentration, and the effect of varying the substrate concentrations are shown in Fig 39 (b,c). Both relationships appear to be essentially linear. The limit of digestion with 20µg of collagen was not reached in these experiments. In addition to this, the telopeptidase activity of Fraction A, appeared to be stable at 4°C, in chromatography buffer (containing Brij 35) for at least 9 months. The activity in Fraction B also appeared to be stable for at least 6 months. The proteinase preparations appeared to be stable in a variety of materials including, glass cylinders, polycarbonate centrifuge tubes, micro-centrifuge tubes (polypropylene) as well as 5ml sterile culture tubes (polypropylene), but not in polyethylene scintillation vials. For assay, or incubation purposes both micro-centrifuge tubes and 5ml culture tubes gave identical results, for the release of the labelled telopeptides. In addition, analyses were apparently not affected by Brij 35. However, use of assay buffers of older than 3 weeks, apparently caused an increase in background. It was thought that deterioration of sodium azide may have been responsible.

J. Analysis of Neutral Proteinases From Cultures of Human Gingival Fibroblasts

As a corollary to the investigation on porcine gingival explants in culture, human gingival fibroblasts were examined for their ability to produce telopeptidase activity

(Scott et al., 1983) The conditioned medium obtained from these cultures was concentrated by ammonium sulphate (80 fold), dialyzed, and treated with pHMB. After dialysis against ammonium propionate buffer as described previously, an aliquot (625µl) was incubated with the ^{3}H-collagen (250µg), for 96 hours at 18°C. The products were fractionated on Bio-Gel P10, and analyzed as described previously. The relative concentration of hydroxynorleucine decreased from 127dpm/nmole of hydroxyproline to 43dpm/nmole of hydroxyproline, or a 66% reduction, without any significant amount of hydroxyproline being released in the low molecular weight fractions (Vt).

The telopeptidase activity of these fibroblast cultures was examined for certain properties. The pH optimum was shown to be between 7.0-8.0. In addition only EDTA but not NEM, PMSF, soybean trypsin inhibitor, or α_1 anti-trypsin inhibited the telopeptidase activity as was described for the telopeptidases from gingival explant cultures. Further analysis of this proteolytic activity was not possible. The cells apparently stopped producing proteolytic activity after the eighth passage. Cells that had been frozen down at the sixth passage and brought back, were also shown not to produce the telopeptidase activity (or collagenase and Azocoll digesting activities).

IV. DISCUSSION

A. Development of a Telopeptidase Assay

The development of a relatively simple assay for telopeptidase activity was crucial for this study. This assay is based on the fact that collagen from tissues such as skin, and which is extractable in acid, contains a significant number of lysinal residues. Since lysyl oxidase probably has no effect on lysyl residues within the helix (Bornstein and Traub, 1979), treatment of this type of collagen with ^{3}H-sodium borohydride should specifically label the telopeptide regions with a stable radioactive marker. Hydrolysis of the ^{3}H-collagen with acid or base resulted in several ^{3}H-labelled products (Fig 11). The main one was the reduced cross-link precursor hydroxynorleucine (and the HCl-derived chlornorleucine). Incubation of the reduced collagen with proteinases, with a specificity towards the telopeptide regions (at a site proximal to the cross-link precursor), resulted in the release of low molecular weight peptides containing ^{3}H-hydroxynorleucine. A maximum of 66-68% of the total content of this reduced cross-link precursor was found to be released. This is comparable with the results obtained with cathepsin D, which has been demonstrated to be specific for the C-terminal extra-helical region on Type I collagen, (primary cleavage site is between residue C-6 and C-7 of the α_1 chain; Scott and Pearson, 1981). It is therefore likely that a significant proportion (up to 30%) of this labelled

amino acid was located at the N-terminal extra-helical region. However, due to the location of the methionines near to the N-terminus (see Fig 2), the cyanogen bromide peptides containing the reduced cross-link precursor of this region (α_1CB0,1 and α_2CB1) would be of low molecular weight (approximately 1,000) and thus not observed on polyacrylamide gel electrophoresis. This would also be true of any intramolecular cross-links situated at the N-terminal telopeptide region (aldol condensation product). There was only one major ^{3}H-labelled peptide found (by polyacrylamide gel electrophoresis) in a cyanogen bromide digest of the labelled collagen (see Fig 32). This peptide had the relative mobility of α_1CB6a. The partially degraded or shortened form of α_1CB6 which was labelled as α_1CB6b, is unlikely to contain the lysinal residue (residue C-17). This conclusion was based on the molecular weight calculated from the electrophoretic scans (1,200 dalton less than that of α_1CB6a), and on the negligible amount of radioactivity associated with the α_1CB6b. The C-terminal residue of α_1CB6b could be C-15 or C-16, and thus this peptide could still be a substrate for a C-terminal telopeptidase activity.

The reduced collagen was not, however, an ideal substrate for a telopeptidase assay. There was a significant amount of radioactivity (80% of the total) associated with the helix. This was labile on acid and base hydrolysis, as well as to the treatment in 70% formic acid during cyanogen bromide digestion. Release of some of this label during

incubation of the substrate and enzyme preparation to be tested, would give spurious results in the assay. This was believed to have occurred during the analysis of telopeptidase activity of unfractionated conditioned medium. Conditioned medium that had been dialyzed against assay buffer was found to contain approximately 20-30% less telopeptidase activity than non-dialyzed conditioned medium (results are not shown). However, this may have been due to actual losses on dialysis. A further indication of some problem with the assay when used with crude medium was that a significant amount of 'telopeptidase' activity from conditioned medium was not precipitable with ammonium sulphate, even at 90% saturation. Nevertheless, the activity that was recovered, was 'telopeptidase'. There was a direct correlation between the level of telopeptidase activity in Fraction A (the pooled fractions from gel filtration chromatography containing the telopeptidase activity of apparent Mr of 70,000), as determined by the assay using the ^{3}H-collagen, and the observed effects of this activity on acid soluble collagen (percent of the α_1CB6 that was shortened). In other words, the assay appeared to be reliable once the proteinase was partially purified. Improvements to the assay could be directed towards decreasing the non-specific label. A shorter period for reduction may have helped. Dialysis of the reduced collagen against sodium borate buffer pH 9.1, removed 70% of the label that was not initially removed with dilute acetic

acid. Increasing the pH of the buffer may remove additional radioactivity, however soluble collagen becomes progressively more unstable at higher pH, thus care would have to be taken not to cause denaturation.

B. Proteinases with an Activity Directed Towards Collagen

Proteinases with an Activity Directed Towards the Telopeptides

Previous investigations into the effects of various proteinases on native collagen (other than at the collagenase cleavage site) have essentially focussed on the N-terminal telopeptide region (see Burleigh, 1977). An effect on this region of collagen (if cleavage occurs proximal to the cross-link site) can easily be demonstrated by the difference in the ratio of β to α chains on polyacrylamide gel electrophoresis. As described in the Introduction, the majority (if not all) of these β components are likely derived from intramolecularly cross-linked α chains.

To demonstrate the removal of the C-terminal extra-helical region requires a more rigorous approach than for the N-terminal telopeptide. One of the problems overcome initially was the isolation of collagen with an intact C-terminal extra-helical region. This region of the collagen is apparently more susceptible to proteolytic digestion than the N-terminal telopeptide, during the extraction and purification of collagen from the tissues (Stoltz et al.,

1972, 1973). The majority of studies which examined the effects of various proteinases on collagen involved collagen isolated under conditions where no special precautions were taken to avoid this proteolytic degradation. In this investigation acetic acid soluble collagen was isolated in the presence of several proteinase inhibitors. Even with this precaution, not all of the collagen had an intact C-terminal telopeptide (approximately 30-40% of the α_1CB6 was in the b and c forms). This degradation was even more prominent in fetal bovine skin that had been stored frozen for 2 years (50-55%).

Investigations from this laboratory have demonstrated that the C-terminal telopeptide of acid soluble collagen (Type I) is removed by the action of cathepsin D (Scott and Pearson, 1978a), cathepsin B and leukocyte elastase (Scott and Pearson, 1983). Cathepsin B and leukocyte elastase have also been shown to remove the N-terminal extra-helical region (reviewed by Burleigh, 1977). In addition, these proteinases were shown to release collagen monomers (or aggregates) from acid insoluble collagen (Burleigh et al., 1974; Starkey et al., 1977; Scott and Pearson, 1978b). If the incubation temperatures were kept sufficiently below the denaturation temperature of the collagen monomers, there was no apparent attack on the helix. These proteinases however, may have a limited (though important) role in collagen turnover *in vivo*. Leukocyte elastase, a neutral serine proteinase of molecular weight 25,000 (Barrett and McDonald,

1980), may only be present in significant concentrations extracellularly in inflamed tissues. In this investigation only non-inflamed attached gingiva (by visual examination), was excised and cultured. In addition, since leukocyte elastase is known to be a serine proteinase, EDTA should not have affected its activity. The telopeptidase activities in both Fraction A and Fraction B (the pooled fractions from gel filtration chromatography which contain the telopeptidase activity of apparent Mr of 35,000), were inhibited by EDTA, but not by PMSF (specific for serine proteinases).

The cathepsins with an activity against Type I collagen have acid pH optima. Thus in the extracellular matrix, these proteinases may only be functional in situations where a microenvironment at acid pH is established (for example adjacent to cell membrane). This may be the situation during phagocytosis of collagen, in which collagen fibrils have to be cleaved by the cell for endocytosis. On the other hand, Melcher and Chan (1981) suggested that periodontal ligament fibroblasts may release neutral proteinases into the extracellular matrix. These could cleave fibrillar collagen and thus facilitate phagocytosis.

The Telopeptidase Activity in Fraction A

In this investigation a neutral metalloproteinase of apparent Mr of 70,000, with the capability of removing the C-terminal telopeptide of collagen was isolated. It was concluded that this enzyme did not cause cleavage in the

N-terminal telopeptide, proximal to the cross-link site. An activity that removes the telopeptide regions of collagen, as described above for the cathepsins and leukocyte elastase, is expected to release collagen monomers from fibrillar collagen. While an activity directed towards both ends of the collagen molecule may allow for the fastest rate of solubilization, the collagen may still be solubilized by cleavage at the C-terminal telopeptide alone (proximal to the cross-linking site), as was demonstrated for cathepsin D (Scott and Pearson, 1978b).

In incubations of Fraction A with fibrillar collagen, a significant amount of collagen was released in a salt soluble form, of which the major portion (greater than 90%) had not been affected by collagenase. It was shown, by analysis of a cyanogen bromide digest of the solubilized collagen, that a significant proportion of this collagen had lost the C-terminal telopeptide. However, these results would also imply that not all of the collagen molecules need be shortened at this region in order to be released. From these results, all that appears to be required is cleavage (at the telopeptide region) of only about one in five collagen molecules. This is supported by the finding of large molecular weight aggregates of collagen that were observed on polyacrylamide gel electrophoresis of the solubilized products (strong Coomassie Blue staining on top of the 5% polyacrylamide gels).

The Telopeptidase Activity in Fraction B

The region of the collagen molecule (amino or carboxy-termini) that the telopeptidase activity of Fraction B was directed towards was not determined. Incubation of Fraction B with the ^{3}H-collagen, under conditions that caused significant losses of enzyme activity (dialysis and incubation in ammonium propionate buffer containing no sodium chloride), resulted in the release of 38% of the ^{3}H-hydroxynorleucine. Since there was a negligible amount of hydroxyproline in the Vt fraction, the above results would indicate that the proteolytic activity is a true telopeptidase. However, as discussed previously, only 64-68% of the ^{3}H-hydroxynorleucine could be located at the C-terminal extra-helical region. Thus a release of only 38% may be caused, completely or in part, by an activity directed towards the N-terminal telopeptide. In addition, incubation of Fraction B with the other collagen substrates ('cold' acid soluble and insoluble collagens) resulted in essentially uninterpretable data. This is discussed in more detail below.

The Products of the Incubation of Conditioned Medium and Collagen

Initially it was calculated that to obtain the release of 75% of the telopeptides from 100μg of collagen in 24 hours, approximately 3 units of the telopeptidase activity was needed. Based on the relative ratio of collagenase to

telopeptidase activity in Fraction B (690/1), there would be a huge excess (500 fold) of collagenase activity. In other words, in an experimental situation, when there was sufficient telopeptidase to digest 75μg of collagen (removal of telopeptide) there was enough collagenase to degrade more than 50mg of the substrate. In addition, the presence of other proteolytic activities (as demonstrated by the degradation of Azocoll and azocasein) which are due, at least in part, to separate enzyme activities, may have also contributed to the extra cleavages of the collagen products leading to loss of α1/4 fragments and shortening of the α3/4 fragments. Thus as described, it was impossible under these conditions, to demonstrate unambiguously which telopeptide was removed by the action of Fraction B.

There are a number of reports which suggest that collagenase cleaves collagen at other sites in the molecule (evidence reviewed by Harris and Cartwright, 1977). Werb and Reynolds (1975) described such extra cleavages of the products of collagenase action. However upon further purification of the collagenase, these were much less evident. In a recent publication, it was demonstrated that a mixture of trypsin and chymotrypsin caused cleavages within the helix of Type I collagen (Ryhanen et al., 1983). These authors investigated the conformational stability of the collagen using the above enzymes as probes. Ryhanen et al. demonstrated that triple helical collagen, even at a temperature of 33°C, was susceptible and the resulting

polyacrylamide gel electrophoretic profiles of the collagen digest were indicative of multiple cleavages. They concluded that the collagen undergoes temporary and local relaxation of the helix and it is these areas that are susceptible to proteolytic attack. In this study, the action of collagenase at 22°C produces 2 triple helical fragments which have reported denaturation temperatures of 32°C and 29°C (Sakai and Gross, 1967). These products even at 22°C, may be susceptible to extra cleavages in the presence of excessive amounts of proteolytic activity.

In addition, there were other problems encountered in this investigation concerning the products of degradation. Examination of the cyanogen bromide digest revealed an unexpected peptide with an apparent Mr of 18,400. This peptide could not be α_1CB6b, since there was approximately twice the amount of this peptide than would have been expected. It must be due to an extra cleavage of the collagen in a region that would cause the products to be similar to the characteristic collagenase-derived peptides (and thus not distinguishable on 5% polyacrylamide gels). The presence of this peptide was unlikely to be due to a shortened form of either of the α1/4 peptides. In the incubation of acid soluble collagen with Fraction B, the majority of the α1/4 peptides were destroyed, however, after cyanogen bromide digestion, the unknown peptide was still present. Neither was it due to a protein in the enzyme preparation, since it was not seen in a gel electrophoretic

profile of the cyanogen bromide digest of the EDTA-inhibited enzyme incubation. From circumstantial evidence it was believed that the relative amount of the unknown peptide was related to the amount of active collagenase in the incubation mixtures. However, this is not proof that collagenase can cleave at a different site on collagen. If this unknown peptide could be separated and isolated from α_1CB6, then amino acid analysis and comparison to other 'known' cyanogen bromide peptides may pinpoint its origin.

C. Fibrillar Collagen Turnover

Effect of Vertebrate Collagenase on Collagen Turnover

As indicated in the Introduction, there is a general disagreement in the literature concerning the actual role of collagenase in the turnover of fibrillar collagen. Collagen molecules in the fibrils are axially displaced by an integral multiple of D (670nm) (Bornstein and Traub, 1979). The exact lateral arrangement of the molecules within the fibrils (50-100nm in diameter) has not yet been positively established. While intermolecular cross-linking does stabilize this arrangement, the formation of the structure depends on the strong electrostatic and hydrophobic interactions of the collagen molecules. This arrangement of the collagen has been demonstrated to obstruct the entry of other macromolecules (Welgus et al., 1980). These authors studied the kinetics of action of vertebrate collagenase on

collagen that was reconstituted into fibrils *in vitro*. They demonstrated that, in fibrils of 50nm and 100nm diameter, only approximately 10% and 6% respectively of the collagen molecules were immediately accessible to the collagenase. This correlates with the proportion of collagen molecules actually on the fibril surface. Only after removal of the 'outer' layers of collagen did the collagenase act on the underlying substrate. While the gap region (the area between two collagen molecules lying end to end) is near to the collagenase cleavage site in an underlying collagen molecule, the C-terminal end of the helix and the C-terminal telopeptide in fact overlie the cleavage site (Fig 40), and thus may act as a physical barrier to collagenase. The rate of collagenase-mediated collagen degradation in a substrate of this nature is only 1/10 or less of collagen in solution. Reconstituted collagen, as used in the experiments of Welgus et al. (1980), is in fact the type of substrate used by many investigators for determining collagenase activity.

The above situation applies equally well to collagen fibrils that are or are not intermolecularly cross-linked. The introduction of cross-links into ^{14}C-labelled lathyritic collagen reconstituted into fibrils by thermal gelation, has been shown to drastically reduce the rate of degradation by collagenase (Vater et al., 1979a). Incubation of collagenase (sufficient enzyme to degrade up to 35% of the reconstituted collagen) for up to 12 hours at 37°C, resulted in a negligible release of radioactivity (up to 2.4%) from a

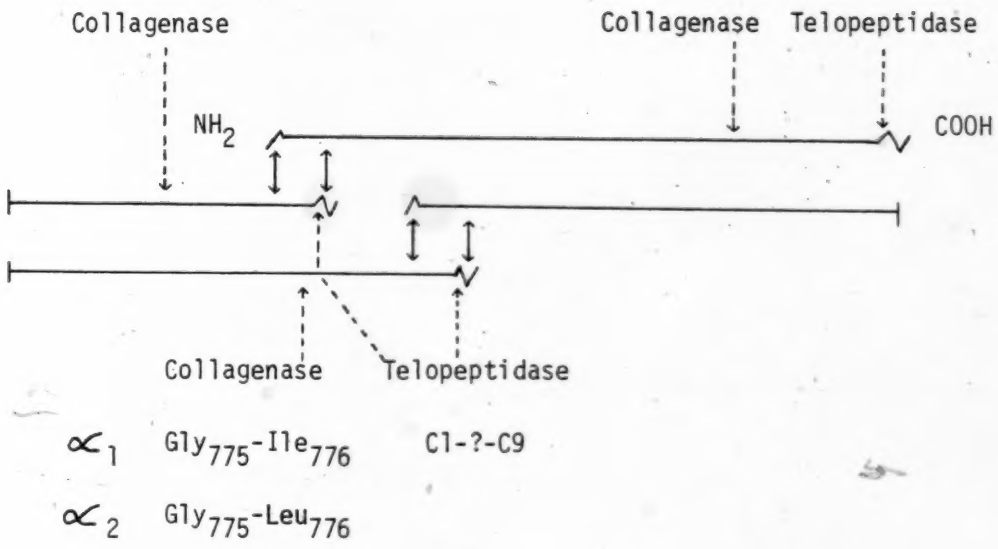


Figure 40. A Schematic Representation of the Location of the Sites on Collagen that are Cleaved by Collagenase and Telopeptidase.

substrate that had on average 0.59 Schiff base cross-links per collagen molecule. Further incubation with more collagenase (between 20-70 fold increase in the enzyme concentration and/or time of incubation) did ultimately solubilize significant amounts of the collagen (up to 75% of the substrate). The ^{14}C-labelled substrate that was used obviously did not have sufficient numbers of intermolecular cross-links to covalently link all the collagen molecules. Thus it would be more susceptible to degradation than the fibrillar collagen as it occurs *in vivo*, with a considerably higher level of cross-linking (Scott and Veis, 1976b; Scott and Edwards, 1981). Woolley et al. (1978) incubated polymeric collagen (prepared by 'α-amylase' dispersion) with collagenase that was shown to be homogeneous by gel electrophoresis. These authors demonstrated that, while this substrate was degraded, the rate was only 5% of that using non cross-linked reconstituted collagen. Nevertheless these authors concluded that collagenase by itself was capable of solubilizing the fibrillar collagen.

Vater el al. (1979a) examined the residual collagen fibrils after removal of the soluble, collagenase-derived, products. They did this in order to determine whether the cross-linking prevented access of collagenase to the cleavage site, or whether hydrolysis could still take place but the cleaved fragments were not released. The collagen residue which was dissolved in acetic acid, or solubilized with treatment with pepsin, was electrophoresed on

polyacrylamide gels. They found no indication of any of the characteristic collagenase products and interpreted this as a lack of effect of collagenase on the cross-linked collagen. However, as described above, in a substrate of this nature, only a small proportion of the collagen molecules would be accessible to collagenase and the methods used may not have been sufficiently sensitive to detect an effect. Nevertheless, the overall data reported by Vater et al. (1979a) strongly suggests that solubilization of fibrillar collagen may require other proteolytic activity(s), such as the one described in this investigation.

Other Factors that May Modulate Collagen Turnover *In Vivo*

Fibrillar collagens (from human diaphragm tendon), isolated from 'older' tissues are apparently more resistant to the action of bacterial collagenase (Hamlin and Kohn, 1971; Hamlin et al., 1980). Miyahara et al. (1982) compared the relative resistance to pepsin degradation of isolated human skin fibrillar collagen from an infant, a 32 year old and a 72 year old. Approximately 60% and 90% of the insoluble collagen from the 32 and 72 year old respectively was resistant (did not solubilize), however essentially all of the fibrillar collagen from the infant was solubilized with pepsin treatment. One possible reason for this is the increase in non-reducible intermolecular cross-links that occurs with maturation (Light and Bailey, 1980). There do

not appear to be any reported studies of the action of vertebrate tissue collagenase on fibrillar collagen of various ages. However Sopata et al. (1974) demonstrated that reconstituted collagen fibrils that were 'matured' *in vitro* (at 37°C) for periods from 5 hours to 10 weeks showed an increased resistance to degradation by collagenase from polymorphonuclear leukocytes.

There may be other factors which are involved in the modulation of collagenase activity *in vivo*. Certain extracellular components such as proteodermatan sulphate and fibronectin have been demonstrated to interact with collagen (see Introduction). J.E. Scott (1980) presented electron microscopic evidence for an interaction of proteodermatan sulphate with fibrillar collagen (using a stain for sulphated glycosaminoglycans). The proteoglycan appeared to be associated with collagen at regular repeating intervals along the fibril in areas that corresponded to the 'gap region'. An interaction of this nature may impede entry of proteinases such as collagenase into underlying regions. Fibronectin has also been shown to interact with collagen, in *in vitro* experiments, specifically in the region containing the collagenase-binding region (Kleinman et al., 1981). In addition, fibronectin has been shown to affect the rate of degradation of different types of collagen by collagenase, presumably for the reason described above (Menzel and Borth, 1983). Differences in the rate of digestion of different types of collagen were related to the

relative affinities of the collagen types for fibronectin. There are several studies which have demonstrated proteoglycan degrading activities in cultures of connective tissues (Dingle, 1975; Galloway et al., 1983). Thus removal of these macromolecules may be a prerequisite for the degradation of fibrillar collagen. However there is no direct evidence that proteoglycans reduce the rate of collagenase mediated collagen degradation. Woolley and Evanson (1977) incubated the proteoglycans isolated from human and porcine cartilage, with reconstituted collagen, and found that collagen breakdown by rheumatoid synovial collagenase was unaltered.

In this Department, monoclonal antibodies towards the core protein of proteodermatan sulphate, have been developed by G.A. Pringle. With these antibodies it was demonstrated that acid soluble collagen isolated as described in the Methods, contained proteodermatan sulphate (or at least the protein core) at an approximate concentration of 0.5% (w/w; on a molar basis it was approximately 2%). The acetic acid insoluble collagen used in this study was not analyzed, but it quite likely contained small but possibly significant levels of this proteoglycan. Proteodermatan sulphate isolated by extraction with 4M-guanidinium chloride from bovine dermis and purified, as described by Pearson and Gibson (1982), was completely degraded by proteolytic activity of Fraction A and to a lesser extent by Fraction B. However, as indicated above, the proteodermatan sulphate was

extracted under denaturing conditions, and any conclusions based on the degradation of this material may not be physiologically meaningful.

An important factor related to the turnover of fibrillar collagen is the temperature dependence of the collagenase mediated breakdown. Harris and McCroskery (1974) observed that the degradation of Type II fibrillar collagen was increased 4-fold by increasing the temperature of incubation from 33°C to 36°C. Welgus et al. (1981b) studied the 'activation' energy of human skin fibroblast collagenase and demonstrated that increases of 2°C in the incubation temperature caused a 3-fold increase in reconstituted collagen breakdown. In addition these authors demonstrated that the substitution of D_2O (at 90% concentration) instead of H_2O in the incubation mixture caused a 9-fold decrease in the rate of solubilization. Welgus et al. concluded that water is involved at the rate-limiting step of collagen breakdown, and that with increases in the temperature of incubation the collagen fibrils would become more accessible to water.

D. Purification of the Neutral Proteinases

The reports of both Woolley et al. (1978) and Vater et al. (1979a) essentially demonstrated opposite views about the function of collagenase, however there was one similarity in their results. These 2 groups of investigators both concluded that collagen containing intermolecular

cross-links was degraded at a much slower rate than non-cross-linked fibrillar collagen. The collagenase preparation utilized by Woolley et al. may have been contaminated with a 'telopeptidase' as may that of Vater et al. The presence of a telopeptidase activity may have initiated the fibrillar collagen breakdown, as described in a later section. The preparations of collagenase used by the above investigators were obtained using a fairly simple purification. In fact, Vater et al. (1979a) had 2 protocols for the preparation of collagenase, one of which just involved concentration and chromatography on agarose A1.5M, after which, these authors claimed the collagenase preparation was free of non-specific proteolytic activity. However, in the present investigation the fractions obtained by chromatography on agarose A0.5M, which contained active collagenase (Fraction B) also contained a significant amount of telopeptidase activity. Cawston and Tyler (1979) purified collagenase using gel filtration, DEAE-cellulose, heparin-Sepharose and zinc-chelate-Sepharose chromatographies. These investigators indicated that although the collagenase preparation was at least 94% pure it still had activity against azocasein. This activity was shown to co-migrate with collagenase on alkaline polyacrylamide gel electrophoresis, and to be inactivated at the same rate as collagenase by heat. A sample of this preparation of collagenase (kindly supplied by T.E. Cawston), was demonstrated to contain a significant level of telopeptidase

activity (see Table 12). The ratio of collagenase to telopeptidase activity was in fact similar to that observed in Fraction B.

Several attempts were made to separate the proteolytic activities in Fraction B (Mr of 35,000). The protocol of Cawston and Tyler (1979) as described previously, was followed (performed prior to the analysis of the telopeptidase activity in the collagenase preparation of Dr Cawston). As indicated previously, the telopeptidase activity with apparent Mr of 35,000, was not separable from the collagenase by this protocol. In addition, Fraction B was chromatographed on DEAE-Sepharose (to remove strongly anionic macromolecules) and then chromatographed on a column packed with pepsin-treated-collagen bound to CNBr-activated Sepharose 4B. The pepsinized collagen was essentially devoid of all of the C-terminal telopeptide regions. Thus, the theory behind this approach was that the telopeptidase activity would be unretarded, whereas the collagenase should bind. This approach also did not succeed for reasons already alluded to in the Results and which are discussed below.

Fractionation of proteinases by affinity chromatography was not, on the whole, very successful in this investigation. One of the major problems concerned recovery of the proteolytic activity after dialysis against buffers containing no sodium chloride. As discussed by Nagai and Hori (1972) and Harris and Cartwright (1977), significant losses of collagenase activities are sometimes inevitable

during dialysis under these conditions. Loss of proteolytic activities in this investigation was on occasion close to 100%. In the later stages of this investigation, Brij 35 was added to all the chromatography and dialysis buffers. This was included to decrease the risks of proteinase adsorption or precipitation, as recommended by Cawston and Tyler (1979). However, recoveries of proteolytic activities were still somewhat variable when dialysis was performed against buffers containing Brij 35, but with no sodium chloride. When an aliquot of Fraction A was dialyzed against ammonium propionate buffer, in preparation for the demonstration of removal of labelled telopeptides by amino acid analysis, essentially all of the activity was lost.

Another factor which probably influenced the separation and recovery of the proteolytic activities was the scale on which the various purifications were attempted. A number of purification techniques for collagenase, that have been reported, appear to require very large quantities of conditioned medium (10-100 litres; Stricklin et al., 1977; Cawston and Tyler, 1979; Roswit et al., 1983). This amount was necessary to minimize the losses of proteolytic activity due to handling, assay, and non-specific adsorption of the proteinases. Ion-exchange chromatography, a technique used by some investigators to purify collagenase, has been described to result in relatively large losses of proteinase activity (Harris and Vater, 1980). Stricklin et al. (1977) used phosphocellulose chromatography and recovered only 34%

of the collagenase activity. Woolley et al. (1978) recovered 15% of the collagenase activity after chromatography on QAE-Sephadex. Other purification protocols such as those involving affinity chromatography may also result in large losses of activity. Bauer et al. (1971) chromatographed 5-10mg of partially purified human skin collagenase on a column packed with 2.5ml of collagen-Sepharose and recovered only approximately 15% of the collagenase activity. However this low recovery of activity may, in part, be due to the preparation and concentration procedures (dialysis and lyophilization).

In the early stages of proteinase fractionation in this investigation it was thought that any attempts at purification should be performed using relatively small amounts of enzyme and then only scaled up when the given technique was proven. It was thought that the assay techniques were sensitive enough to detect the proteolytic activities even if large losses occurred. Initially the proteinases, from 50-100ml of conditioned medium, and which were precipitated with ammonium sulphate, were applied to the collagen-Sepharose columns. In a later study, after gel filtration, the equivalent of 250ml of conditioned medium (Fraction B), which contained approximately 100μg of protein, was dialyzed and applied to the columns. Later purification steps may have been more successful if a much larger starting amount of the enzyme had been used.

As described previously, attempts to fractionate the activities in Fraction B did not succeed. Thus it cannot be directly concluded from these experiments that the telopeptidase activity of Fraction B was not due to an additional activity of collagenase. However, in an earlier experiment which involved the affinity chromatography (on collagen-Sepharose) of conditioned medium, an EDTA-inhibitable telopeptidase activity of an apparent Mr of 25,000-35,000 was recovered without a trace of collagenase activity (determined by the collagen-gel capillary assay, incubated for 1 week). It therefore seems likely, even though this experiment could not be repeated, that the telopeptidase activity in Fraction B was due to a proteinase distinct from collagenase.

E. Latent Collagenase

There was a significant amount of latent collagenase in Fraction A after initial chromatography of the concentrated conditioned medium on agarose A0.5M. The amount of this latent collagenase however apparently decreased (in relation to the telopeptidase activity) with each subsequent gel filtration step. This decrease was related to the appearance of active collagenase in those fractions of apparent Mr of 35,000. It seems likely that this latent collagenase was a complex of some nature that was relatively resistant to the treatment with trypsin (both soluble and insoluble) and to pHMB. It was not dissociable at either high (1.0M-NaCl) or

low (0.2M-NaCl) salt concentrations. Cawston et al. (1981) isolated an inhibitor of collagenase from cultures of rabbit bone which was given the name of TIMP (tissue inhibitor of metal-dependent proteinases). The inhibitor, of 28,000 molecular weight, interacts with rabbit bone collagenase in a stoichiometric 1:1 ratio. This complex was not dissociable by treatment with trypsin or the organomercurials.

Macartney and Tschesche (1983a,b) isolated an inhibitor from human plasma (glycoprotein of 28,000 daltons) which was apparently identical to the β_1-anticollagenase described by Woolley et al. (1976b). This inhibitor also formed a tight complex with collagenase (human skin fibroblast) in a stoichiometric 1:1 ratio. However, this complex was dissociable by treatment with compounds containing disulphides (eg cystine, insulin, TLCK-trypsin) and by mersalyl (which is an organomercurial).

The above types of inhibitors have been studied by numerous investigators, however the findings that have been reported do not appear to explain the latency and the activation that were observed in this study. From the data it is believed that there was an equilibrium between the enzyme-inhibitor complex and free enzyme and inhibitor. While the complex was resistant to dissociation by proteolytic and chemical means, the free inhibitor may not have been stable to these treatments. Inactivation of purified tissue inhibitor by proteolytic means has been documented (Vater et al., 1979b). This would explain the

increased amount of collagenase activity in Fraction A after treatment with soluble trypsin and also explain why there was not total conversion of the latent enzyme to an active form. If the inhibitor was destroyed by trypsin then the equilibrium between free and bound enzyme would have to be re-established. Thus more free active collagenase would be present. An equilibrium hypothesis would also explain why there was no active collagenase in Fraction A, as determined by the collagen-gel capillary method, immediately after the chromatography of Fraction A on agarose A0.5M, whereas after several weeks at 4°C active enzyme was demonstrated.

Incubation of Fraction A (after the third chromatography) with acid soluble collagen at 22°C, for 29 hours resulted in a low but significant amount of the characteristic collagenase products. As described previously incubation of Fraction A with reconstituted collagen at 37°C, demonstrated collagenase activity only after 1 week. The explanation for this is unclear but the results suggest that the latent collagenase was activated more rapidly in the presence of soluble collagen than with reconstituted collagen. This implies also that the equilibrium between free and bound (to inhibitor) collagenase is disrupted by competition with the substrate.

Vater et al. (1979b) studied the effects of an inhibitor, isolated from cultures of rabbit tendon, on the ability of collagenase to degrade collagen in solution (monitored by changes in viscosity). These authors reported

that when the collagenase and inhibitor were added to the collagen, there was a delayed response before inhibition was noted. However this delay was abolished when the collagenase and inhibitor were pre-incubated. In addition, the inhibition was not complete. These results may be interpreted in the light of the above to suggest that there was a competition between inhibitor and substrate for the enzyme and also an equilibrium between free and inhibitor-bound enzyme.

There may be another explanation for some of the observations. Polymorphonuclear leukocytes are known to contain in their 'specific granules' a collagenase of apparent Mr 60,000 (Robertson al., 1972a,b; Murphy et al., 1977). This collagenase acts at the same site on collagen as the tissue collagenases. However this enzyme has been reported to be not very effective in solubilizing reconstituted collagen fibrils (Lazarus, 1972). Based on this information a number of investigators analyzing leukocyte collagenase use an assay involving collagen in solution. However, some investigators do use reconstituted collagen fibrils as the substrate (Robertson et al., 1972a,b; Sopata et al., 1974). Thus it is not clear whether the assay used in this study (collagen-gel capillary assay) would show leukocyte collagenase activity. Although in our investigation, care was taken not to include inflamed tissue in the cultures, some contamination by leukocytes was probably inevitable. Nevertheless, it seemed unlikely that

there was a significant contamination with leukocyte elastase (this enzyme, which is a serine proteinase, can act as a telopeptidase (Starkey et al., 1977; Scott and Pearson, 1983)) since in this investigation addition of EDTA abolished the telopeptidase activity of conditioned medium. This did not completely rule out contamination with leukocyte collagenase. The elastase is located in the azurophilic granules (Dewald et al., 1975), while the collagenase is found in the specific granules (Robertson et al., 1972a,b; Murphy et al., 1977). These granules are apparently released at different rates from the leukocytes, in response to different stimuli (Gallin and Wright, 1978; Dewald et al., 1980).

The presence of collagenase in Fraction A was a problem which was not solved in this investigation. It is possible that other methods of activation such as treatment with chaotropic agents (KSCN or NaI) or agents such as those described by Macartney and Tschesche (1983a,b; see above), may have been more effective than trypsin. Alternatively a longer exposure to soluble trypsin could have been used. In this laboratory exposure of unfractionated medium to trypsin-agarose for 2 hours was shown to result in a very small increase in activity over that seen at 1 hour, but at 3 hours the level of activity appeared to decrease. Latent collagenase was still seen after 2 hours of exposure to the trypsin-agarose. Demonstration of whether leukocyte collagenase was actually present in Fraction A would be very

difficult. Other than by employing a specific antibody to this collagenase, there may be no solution (in a recent report, Hasty et al. (1984) demonstrated that a monoclonal antibody for neutrophil collagenase, which caused significant inhibition of activity, did not cross-react with collagenases from human skin fibroblasts or human rheumatoid synovium). If complete removal of all latent collagenase cannot be achieved, then there will always be a doubt about the origin of that which remains.

F. Collagenase Assays

One of the problems that hampered this study in the end stages was the lack of a rapid and reliable collagenase assay such as those using a radiolabelled or other type of labelled collagen. One of the initial worries concerning some reported collagenase assays was that the telopeptides could also be labelled and removal of these would give spurious results. In addition, since telopeptides are known to be involved in collagen fibril organization, prior removal of these will make the fibrils (if a fibril assay was utilized) less stable (see Introduction). In this investigation attempts were nevertheless made to use ^{14}C-acetylated collagen. However all of the attempts, using a variety of techniques, failed (background values in most cases were too high). One possible explanation is suggested by the findings of Fujimoto (1970). This author demonstrated that such collagen contains both N- and O-acetyl groups.

Both the shrinkage temperature and the resistance to digestion by pronase were lower than that of either native collagen or collagen with only N-acetyl groups. In addition, increasing the level of O-acetylation increased the susceptibility of the collagen to pronase. The O-acetyl groups can apparently be removed by incubation in sodium carbonate pH 11.4 for 6 hours at 25°C (Fujimoto, 1970). However this was not pursued in this study. The collagenase assay used (collagen-gel capillary method) had the very important advantage over many other types of assays that trypsin had no effect. In other assays reported the digestion due to trypsin is generally 10% or more.

G. Gingival Fibroblast Cultures

The proteinases found in conditioned medium of cultures of gingival explants may be derived from a variety of cell types. In addition, this is a degenerating culture with a significant amount of connective tissue breakdown and release of the degradation products into the culture medium. The presence of these could interfere with purification of the proteinases. Fibroblast cultures from porcine gingiva were established in this investigation, following the technique described by Pettigrew (1978). Several sets of cultures were grown up and tested for production of the proteinases. Conditioned medium obtained from cultures of either a mixture of fibroblast/epithelial like cells, or essentially only fibroblast-like cells, obtained at the

third to seventh passage did not appear to produce detectable amounts of either collagenase or telopeptidase.

Human gingival fibroblasts (Gin-1, American Type Culture Collection) were demonstrated by Hurum et al. (1982) to produce collagenase. In this laboratory, this cell line was demonstrated to produce, in addition to collagenase, a telopeptidase activity that was inhibited by EDTA. This activity was shown to have the same pH optima as was shown for the porcine gingiva explant conditioned medium. Further studies on this fibroblast enzyme however were not possible for reasons already described.

H. The Telopeptidases of Porcine Gingival Explant Cultures

From the evidence presented in this thesis, there is no doubt about the existence of a C-terminal telopeptidase in cultures of both porcine gingival explants and human gingival fibroblasts. The finding of 2 telopeptidase activities of Mr of 35,000 and 70,000 in the explant cultures, may possibly be due to dimerization or aggregation of the 35,000 molecular weight species, even though attempts to disaggregate the 70,000 molecular weight species with trypsin, pHMB, or variations in salt concentration did not succeed. The two telopeptidase activities may be completely different enzymes which could possibly originate from different cell types. It would be of considerable interest to characterize the telopeptidase activity in cultures of human gingival fibroblasts.

It would be most speculative to assume that the telopeptidase activity (primarily of Fraction A) was due to a previously undescribed proteinase. It is likely that the proteinase(s) that has this activity has already been isolated and characterized as a different entity. A neutral metalloproteinase of an apparent Mr of 58,000 (active form), and with an activity against gelatin, was isolated from cultures of mouse bone explants (Vaes et al., 1978). Sellers et al. (1978) described a similar activity in rabbit bone cultures however they reported the size of only the latent form (71,000 daltons). Heath et al. (1982) demonstrated the presence of a latent 'gelatinase' of apparent Mr of 102,000, in cultures of human gingival explants. Sakyo et al. (1983) extracted 2 metal dependent neutral proteinases of Mr of 70,000 from rabbit uterus. One of these appears to have similar properties to the telopeptidase of Fraction A. This enzyme, which degrades ^{14}C-labelled gelatin, had a similar pH optimum, as well as being inhibited by EDTA, but not by PMSF or NEM. However it was shown to be activated with organomercurials (pCMB or APMA) but not by trypsin. In our study, latent telopeptidase activity was activated equally well by either trypsin or pHMB. The latent 'gelatinases' described by Vaes et al. (1978) and Sellers et al. (1978) were also activated by either trypsin or APMA.

I. Potential Role of the Telopeptidase Activities

The role of the telopeptidase activities in connective tissues may be that of initiating the degradation of fibrillar collagen. This does not rule out action of collagenase on this type of substrate. It is possible that the two enzyme activities work hand in hand in potentiating solubilization. Synergism in breakdown of fibrillar collagen has been proposed by a number of investigators. However experimental evidence appears to be limited to the leukocyte proteinases. Sopata et al. (1974) incubated (at 37°C) insoluble human skin polymeric collagen with either a leukocyte homogenate, a partially purified leukocyte collagenase, or rheumatoid synovial fluid. Low levels of solubilization were obtained when these were incubated separately, as well as when the purified collagenase was incubated with the rheumatoid synovial fluid. The extent of digestion was increased 10 fold when both leukocyte homogenate and rheumatoid synovial fluid were incubated together. The authors proposed that an unknown proteinase in a latent form was present in the leukocytes, and that this proteolytic activity was essential for the initiation of fibrillar collagen breakdown. Lazarus et al. (1972) demonstrated that the incubation of neutrophil serine proteinases and the neutrophil collagenase resulted in the synergistic degradation of reconstituted collagen fibrils. The neutrophil collagenase by itself was ineffective. Starkey et al. (1977) purified leukocyte elastase and

cathepsin G and demonstrated the solubilization of insoluble Type II collagen. They proposed that the elastase (and/or cathepsin G) attacked the terminal telopeptides of the cartilage collagen, which then became soluble and susceptible to collagenase cleavage. In addition to the effects of the 'telopeptidase' activity of elastase, in the presence of collagenase, there may be a third important component in the degradation of collagen. Synergism in solubilization of various insoluble collagen types (in the form of tissue powders) was demonstrated with leukocyte collagenase and a leukocyte 'gelatinase' (Murphy et al., 1982). The gelatinase activity (neutral metal-dependent proteinase of apparent Mr of 110,000) was isolated from a third set of leukocyte granules (C-particles). It would seem logical that a gelatin degrading activity would increase the overall rate of degradation of the substrate by destroying the collagenase-derived products. These products may interfere in the accessibility of the collagenase to the substrate, or may compete with the native collagen for binding to the enzyme.

In this study the actual roles of collagenase and telopeptidase could not be defined because we did not have a completely pure preparation of either of the activities. In the final experiment, the fibrillar collagen substrate, when incubated with Fraction A and Fraction B, was probably saturated with proteolytic activity. An equivalent amount of collagen was solubilized by Fraction A and Fraction B using

approximately similar amounts of telopeptidase activity. However the amount of collagenase in the incubation mixtures differed by greater than 3 orders of magnitude (2,000 fold). From the minimal amount of the characteristic collagenase products obtained in the incubations with Fraction A, it can be deduced that the telopeptidase activity was important for the solubilization. Incubation of a smaller amount of Fraction B (containing the equivalent amount of collagenase as that in Fraction A), would probably have reduced the overall level of solubilization.

J. Conclusion and Suggestions for Further Studies

Collagen metabolism is an integral component in the homeostasis of connective tissues. What initiates the degradation of fibrillar collagen and which physiological factors regulate the process are questions that interest many investigators in the field. However much of the current research efforts is directed towards collagenase in terms of inhibition and activation (of latent enzyme) or of production by various cells. Studies on the other connective tissue proteinases for the most part have been quite limited. One reason for this may be the difficulties in analyzing the breakdown products of collagen such as was encountered in this study. There is still a great deal of research in progress on the biochemical and biophysical analysis of collagen as well as on the interactions of collagen with other extracellular matrix components. In this investigation there were several interesting observations which may only be explained by continued research in this field.

In this investigation a primary objective was to determine whether an activity directed towards the telopeptide regions of collagen was produced by connective tissue cells. As described, such an activity does exist, however there are many unanswered questions. The main one was - what are the actual roles of collagenase and telopeptidase in the initial breakdown of fibrillar collagen? The observations from this investigation offer no

direct proof that the telopeptidase activity is the first step, or that without this activity, collagenase would be ineffective. However based on the results from this study and from others it is likely to have an important role.

It was encouraging to demonstrate a telopeptidase activity from cultures of human gingival fibroblast cells. Purification and separation from collagenase may be much simpler using a system of this nature compared to working with cultures of explants of connective tissues. There should be much less contamination with degradation products of the extracellular matrix, cellular debris and inhibitors. Biochemical analysis of the telopeptidase from fibroblast cultures would determine whether both or only one of the two activities present in cultures of gingival explants is derived from the fibroblasts.

Once the proteolytic activities (collagenase and telopeptidase) are totally separated, then synergism in fibrillar collagen degradation could be properly investigated. If these proteinases were separated from other 'non-specific' proteinases (gelatinases), then incubation of fibrillar collagen at physiological temperatures could be performed. While it is believed that collagenase may have some intrinsic 'gelatinase' activity, it may not become apparent under controlled conditions with limited enzyme concentrations.

Further biochemical analysis of the telopeptidase would be a logical future investigation. The approach that could

be followed could be similar to that of collagenase. Definition of the scissile bond or bonds would be one of the first concerns. Further studies along these lines may allow synthetic substrates to be utilized in the investigations. It would be better if a different substrate, other than collagen, could be utilized in the kinetic analysis of the proteinase. Collagen itself is not a good substrate because of its size and its tendency to aggregate even at relatively low concentrations. Even if aggregation at high concentrations of collagen was prevented (by the use of glucose or arginine), the high viscosity of the solution would affect the rate of degradation, and thus the kinetic analysis. A peptide, either isolated or synthetic, encompassing the known cleavage site could be tested. If telopeptidase had no gelatinolytic activity then denatured collagen with intact C-terminal telopeptide could be tested to determine whether it is a substrate. However it is also possible the triple helical conformation is necessary for proper binding and action of the telopeptidase (as has been described for collagenase and for lysyl oxidase acting on collagen fibrils). Another potential substrate is proteodermatan sulphate, however only the native proteoglycan would be of physiological importance.

There are other projects that could be undertaken once the telopeptidase has been well-characterized. Specific antibodies to the telopeptidase could be utilized in localization studies *in vivo*. This may be relevant in terms

of whether the telopeptidase is involved in pathological conditions such as tumor invasion and periodontal disease as well as in normal conditions including wound healing. The development of specific inhibitors to the telopeptidase may be particulary interesting if the level of telopeptidase were elevated in any particular disorder.

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